

How not to fix the farm economy

President Reagan hopes the deficit will persuade Congress that farm programmes cannot be afforded. But a free market and exports will not cure all ills.

IRONY abounded last week as President Reagan vetoed legislation passed by Congress — with the help of his own Republican Senate — to give farmers emergency credit assistance. Reagan told Congress “someone must stand up to those who would say, ‘here’s the key to the Treasury — just take as many of those hard-earned tax dollars as you want’”.

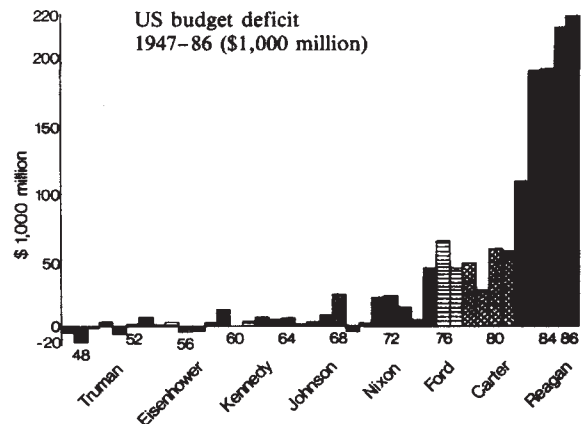
For a President who four years ago handed those keys to Secretary of Defense Caspar Weinberger and has yet to ask for them back despite \$200,000 million-a-year deficits, it was an interesting statement. Amid rhetoric that refers to that unprecedented deficit as some sort of natural phenomenon, it is perhaps easy to forget that it is in fact the direct result of the Reagan tax cuts and defence build-up. A glance at the accompanying graph, prepared by the non-partisan Congressional Budget Office, should be enough to set the record straight.

As for farm credit assistance, Reagan blamed the current troubles on “a generation of failed policies that drove down farm prices and drove up the costs of their land, seed and equipment”, a statement that borders on a *non sequitur*, but which presumably reflects the President’s instinctive dislike for anything that can be traced to Franklin Roosevelt’s New Deal, rather than any precise statement of cause and effect. If the current credit crisis can be traced to anything, it is surely the expansionary policy begun under the Nixon Administration in which farmers were encouraged to plant “fencerow to fencerow”. Farmers expanded; rural banks then pressed farmers to take full advantage of rising land values to borrow to the hilt. Farmers, straddled with high interest rates and coming out of several consecutive years of losses, cannot now get operating loans, and rural banks, having made questionable loans in the 1970s, are faced with insolvency; but the crisis cannot be blamed on a “generation” of government intervention — rather, the blame is with an ill-thought-out burst of *laissez-faire* enthusiasm under Nixon that Reagan is repeating under the rubric of a “market-oriented” policy. The lesson of the 1920s, when *laissez faire* reigned, was that overpopulation is a chronic, recurrent disease in agriculture. Then, as now, the salvation of US farmers was supposed to be exports. The lesson has not apparently been learned that the world does not want to be fed by the United States, except in dire emergency.

It is tempting to view the current farm crisis as the inevitable consequence of an economy undergoing “restructuring”; the end result, in this happy view, is a leaner, more efficient economy. That is the view of David Stockman, director of the Office of Management and Budget, at least. That view is reinforced unwittingly by those proponents of assistance who say the government must help to preserve the “way of life” of the family farmer. An uncritical look at statistics also suggests that small farms are inefficient and might well vanish. But this is a pitfall. It is necessary to understand that the statistics call anything a “farm” that has an annual output of agricultural product worth \$1,000. That definition pulls in thousands of farms that are really not; some 70 per cent of all “farms” have sales of less than \$40,000 per year, and these are about evenly divided between those owned by people who work elsewhere and farm mainly for amusement and those owned by older farmers who paid off their mortgages long ago. They are inefficient and produce a negligible amount of the nation’s food. They are not the people in question.

Those who are calling for help now are the full-time family

farmers, who, according to a 1981 Department of Agriculture study, are virtually as efficient as the very largest farms. (The study found that almost all economies of scale are attained by farms of about 300 acres, roughly the national average.) Expansion, and the inevitable elimination of some operators, occurs not because the smallest are inefficient, but because the smallest simply cannot produce enough income to support a family. Society does not gain from further expansion and concentration of production in fewer hands among this group. Yet that is the inevitable consequence of policies that foster overproduction, depressed prices and the substitution of capital for labour without any gains in overall cost efficiency.



There are really only two points which argue for continued government intervention. One is that society has a legitimate interest in preserving diversity, both geographical and economic, in the farm economy, so long as efficiency is not harmed. Second, again as the 1920s should have taught us, is that periodic booms and busts are harmful and inevitable in an unregulated market made up of producers with a high capital investment who sell what economists call perfectly competitive goods. Farmers are price takers; they must accept whatever the wholesaler offers. And even when those prices fail to meet costs, farmers have little choice but to continue producing (exacerbating the overproduction that depressed prices in the first place) in the hope of making good on their sizeable capital investment. General Motors can stop buying parts when demand falls; Sears can stock new items in its warehouses; but a farmer can do only one thing with a milking parlour.

The Reagan Administration is correct in saying that the current mess of contradictory farm programme has to go. A rational public policy aimed at intervening in the farm economy to level out the peaks and troughs need not cost a sum that approximates total net farm income (as it has in recent years). It certainly does not need programmes that encourage production.

First, instead of throwing up its arms and shouting “compete”, the government could limit subsidies to the first increment of production per farm; the current system of “target prices”, which operates at the margin, is an out-and-out production incentive that has no place in a rational solution. The second, more drastic, step is to be tougher in tying *all* federal assistance to a farmer’s acceptance of rigid production limits. Farmers resist this, as a



matter of pride and to hold to their cherished image of themselves as rugged individualists. The New Deal programmes that have been inherited were attempts to plan the farm economy by doing everything but; it is time to drop the pretence and save a few billion dollars in the process.

Agricultural research, which is a metaphor for the sickness of overall farm policy, has also suffered from short-sightedness. Research programmes arise from the mists to appease some local constituency or deal with some emergency. Painfully absent has been a long-term commitment to solving fundamental problems. A genuine investment in basic research in plant molecular biology — which means considerably more than the still-token effort to establish a peer-reviewed grant mechanism for agricultural research — could pay off in lower input costs to farmers by cutting their dependence on energy-linked tillage, fertilization, irrigation and pest-control costs. That in turn could do much to reduce the expansionary pressures on farmers that are now answered by taking out more and more loans and overproducing.

Imbalanced budgets

Next week's British budget could be decisive for the future of British industry and innovation.

CAN governments do anything at all to revitalize an economy fallen on bad times? In a blend of disappointment and despair, this is likely to be the British government's unspoken question about its revenue budget for the year beginning 1 April, due next week. For outwardly the government should now be basking in the rewards of six years of virtue. Learning from the mistakes of its predecessors since 1964, but also for doctrinal reasons, the present government has avoided, almost, direct intervention in the pattern of industry. Consistently it has been saying that governments can hope only to create the climate in which economic growth and thus prosperity will emerge. But eschewing direct intervention, governments must fall back largely on fiscal measures, whence the importance of next week's budget. The worry for the British and their government is that six years of almost consistent budget-management have so far produced only one tangible success — the inflation rate is a modest five per cent a year. Otherwise, unemployment continues to grow while the slow growth of the Gross National Product in the past few years consists largely of the expansion of service industries. Manufacturing industry (apart from oil) is still producing less than in 1979. And now interest rates are higher, and sterling lower, than ever before.

What has gone wrong? Why has so much effort been so poorly rewarded? From the outset, one of the government's key objectives has been to reduce the proportion of the national income spent by local and central governments, in the twin belief that the proportion has become too high and that, if the level of taxation were reduced, people would have more to spend in ways that would stimulate the economy. More recently, the same British government has been saying that direct taxation (on income) should be replaced by indirect taxation (on spending) because people could then choose investment rather than consumption.

Three things have caught the British government napping. First, public spending has remained high as a proportion of the total because of the growing social costs of unemployment and longevity, but also because spending on essential services such as education is so meagre that it cannot easily be cut. Second, when people have extra money because they pay less tax, they (or the financial institutions that hold their savings) are as likely to invest in industry elsewhere as in Britain. That is why there is something in the view of those economists who argue that restraints on capital outflows would bring immediate prosperity. (The snag is that the short-term would be very short.) Third, the government's actions have not been nearly as consistent as its rhetoric. Last month's sterling crisis seems to have been partly triggered by a belief that the government was allowing the money supply to grow in a way contrived not to show up in the accepted monetary statistic—but can it make sense to spend at least £10,000

million to replace Polaris nuclear submarines by Tridents at a time like this? (An Anglo-French accommodation on nuclear weapons would be cheaper and militarily prudent.)

Signs that the British government is beginning to lose its composure are, in the circumstances, inevitable but significant. With the sterling crisis, last month was replete with signs of fraying nerves. Even more remarkable was an attack delivered last Friday by Mr Nicholas Edwards, Secretary of State for Wales, on the financial institutions of the City of London. Mr Edwards accused them of "prejudice, ignorance and a striking lack of awareness" of what is happening in modern industry. He singled out an electrical and defence contractor, the General Electric Co. PLC, for having accumulated large amounts of cash (in excess of £1,500 million) and for turning itself "into an investment bank" rather than investing in innovation. But what else can Mr Edwards expect, when his own government is prepared to offer people a rate of interest as much as ten per cent above the inflation rate, and when the rewards of innovation are unlikely to be as great?

That is the clue to the fiscal part of the solution to the problem of encouraging reinvestment in British enterprise. British interest rates are at present high not merely because they are tied to interest rates in the United States but because of the money mismanagement rumbled a month ago. The way to bring them down, and to cut the inflation rate, is further to reduce public spending. Tax cuts would work in the same direction but less quickly. The government's difficulty is that it has cut most things to the bone already, so that there is little scope for further manoeuvre. Sooner or later it will have to bite the defence bullet, and the sooner the better. Certainly there is little hope that productive investment can be stimulated by devices such as the ill-starred Business Expansion Scheme, which offers exemption from income tax for five-year investments in new business, and which seems chiefly to have stimulated property development. The moral, for those who like their fiscal policies to be clean and tidy (like the present government) are that a belief that the market knows best is incompatible with attempts to rig it. The moral for those who say that Britain is good at invention but poor at exploitation is that the explanation may lie in fiscal mismanagement. □

Polish normalcy?

Is the Polish government serious in saying it wants normal relations with the outside world?

THE account of Polish science by Vera Rich on page 123 says nothing of the circumstances in which the information was gathered. Ms Rich left London for Warsaw three weeks ago, on a visa provided by the Polish Embassy in London. The visa was due to expire at midnight on Tuesday, 26 February. Protesting in London that she planned to return on a charter flight only on the following day, 27 February, Ms Rich was told that there would be no difficulty if she left on the first flight that day.

On the morning of 27 February at Warsaw airport, the first flight was delayed for several hours, until 11 a.m. On exit, Ms Rich was told that her visa was not in order, she was stripped and searched, a number of documents and audio-tapes were confiscated and Ms Rich was bundled into the waiting aircraft. She returned to London in great distress, humiliated and shocked.

The Polish government is understandably ambivalent towards journalists, especially those from abroad. It rightly regards a reasonable trickle of such people as a proof that normalcy has returned, but it cannot apparently stomach, or even understand, journalists' inevitable curiosity about the events of the past four years. Not that Ms Rich's too-brief visit to Warsaw last month was in any way subversive — most of her meetings with officials were arranged under government auspices anyway. The Polish Embassy in London has not yet replied to a request for an explanation of why Ms Rich was bundled out of Warsaw in such an outrageous fashion. This note is meant to jog the embassy's memory. The explanation, if forthcoming, will of course be published. □

Nuclear winter

Pentagon says yes, it may happen, but "so what?"

IN its first official statement on "nuclear winter", the Department of Defense (DoD) has acknowledged the possibility of severe climatic change following a major nuclear war. But the DoD statement, which comes in the form of a report ordered by Congress*, asserts that the only policy implication is to affirm current US strategic doctrine on deterrence, arms control and Star Wars missile defence.

The DoD report echoes an earlier study issued by the National Academy of Sciences last December in its criticism of the large uncertainties in published estimates of the climatic effects of nuclear war. "At this time, for a postulated nuclear attack and for a specific point on the earth", the DoD report says, "we cannot predict quantitatively the materials that may be injected into the atmosphere, or how they will react there . . . we do not expect that reliable results will be rapidly forthcoming."

DoD specifically criticizes uncertainties in the results of the study by Turco *et al.* (*Science* 222, 1283; 1983). It says the estimate of the amount of smoke that would be produced in fires ignited by nuclear blasts was the result of combining a large number of poorly-known variables; "in actuality, the same yield weapon could produce vastly different amounts of smoke over different target areas and under different meteorological conditions", DoD says.

A preliminary DoD assessment of 3,500 "hypothetical" non-urban target locations concludes that smoke production would be 30 times less than the Turco *et al.* assumption for July and almost 300 times less for January**. The DoD report also suggests that the results may have improperly shifted the focus from dust to smoke; if smoke production has been overestimated, and if one assumes a war scenario in which, for example, ground-burst attacks on missile silos predominate, producing a lot of dust, the effects of dust may outweigh those of smoke.

The DoD report also claims that initial results of three-dimensional models constructed by DoD and National Center for Atmospheric Research scientists show that scavenging of smoke and dust from the atmosphere may be "substantial".

DoD is spending \$1.5 million on nuclear winter research this year and has requested \$2.5 million for next year. An additional \$2 million per year is coming from the

Department of Energy.

But whatever the outcome of these studies, DoD is already suggesting that they will have no effect on US strategic policy. DoD claims credit in the report for having already adopted policies designed to limit both the extent of nuclear war and its atmospheric consequences. It claims that US policy is to avoid urban population centres in its targeting plans, a policy that would also limit bomb-ignited fires.

Significantly, DoD also uses the opportunity to push President Reagan's Star Wars plans, noting that even "single-layer defense" — perhaps a reference to a terminal site defence, the one star wars component that may be possible with existing technology — "may provide a greater mitigating effect on atmospheric

consequences than could result from any level of reduction likely to be accepted by the USSR in the near term".

The report dismisses the argument that the Soviets would respond to a US anti-missile defence simply by increasing their offensive forces, claiming that since the signing of the anti-ballistic missile treaty in 1972, the Soviets have increased their forces fourfold. The report does not mention the US decision to arm its intercontinental ballistic missiles with multiple warheads during that period.

The clincher, as far as the Pentagon is concerned, is that even if the United States becomes convinced of the reality of the nuclear winter, "we cannot be confident that the Soviets would expect such effects to occur as a result of all possible Soviet attacks".

The DoD report claims that the Soviets have performed no independent calculation on climatic effects of nuclear war and in fact "show no evidence of regarding the whole matter as anything more than an opportunity for propaganda".

Stephen Budiansky

ASATs

US Air Force slows the pace

Washington

ALTHOUGH the congressional ban on the further testing of the US anti-satellite weapon (ASAT) expired last week, new tests have been postponed at least until June. Technical problems are partly responsible for the delay, but diplomatic concern about the Geneva arms control talks, due to open next week, may also have been influential.

The Air Force officially refuses to discuss the technical problems or the testing schedule of ASAT. But administration officials last week quietly spread the word about the technical delays. And according to *Commerce Business Daily* of 21 December 1984, the Air Force has asked for bids on a contract for the redesign of the ASAT's "maneuvering propulsion package", a group of 57 miniature solid-fuel rockets that steer the 12-inch ASAT projectile into its target.

The contract announcement mentions contamination from motor exhaust as one problem to be corrected. According to John Pike, a space-weapons expert with the Federation of American Scientists, contamination of infrared sensors has been a "canonical problem" with this type of weapon, going back to prototypes developed for ballistic-missile defence two decades ago. Infrared sensors are used to pick up the heat radiated by the target satellite and so guide the projectile, technically called the "miniature homing vehicle". The projectile carries no explosives, but disables the satellite simply by colliding with it.

So far, the Air Force has tested ASAT only by shooting at a point in space. Live targets have not yet been used. (According

to some reports, for the "point in space" test last November, ASAT's infrared sensors were locked on a star or the planet Jupiter.)

Although the congressional ban on testing against live targets has now expired, the administration would still be required to send a lengthy certification to Congress before such a test could proceed. When the time comes, ASAT opponents say, the administration will have a difficult time completing that procedure with a straight face. Certification will require a finding that the test is necessary to avert "irrevocable harm" to national security, that the United States is trying to negotiate limitations of ASATs, that the test would not irreversibly harm prospects for negotiations and that it is consistent with the anti-ballistic missile treaty.

Representative George Brown (Democrat, California), one of the sponsors of the congressional restraints on ASAT testing and one of 102 congressmen who signed a letter to President Reagan last week urging a continuation of the moratorium, has suggested that the technical problems may be a convenient refuge for an administration that wants to keep up a tough image while not directly jeopardizing the Geneva talks. And Pike said, "I assume that, if they thought the safety of the republic was in jeopardy, they'd be working overtime [to correct the problems] but they've adopted a leisurely schedule".

A middle course that the Air Force may be contemplating is a second test against a point in space in June or July, which would not require the certification.

Stephen Budiansky

* *The Potential Effects of Nuclear War on the Climate*, A report to the United States Congress, Secretary of Defense, March 1985.

** *Smoke Production from Multiple Nuclear Explosions in Wildlands*, R.D. Small and B.W. Bush, Pacific Sierra Research Corp. in the press.

US space science

Manned flight to Mars?

Washington

ALTHOUGH the National Aeronautics and Space Administration (NASA) is officially keeping its distance, a dedicated group of space scientists within and without the agency is busily hatching plots for a manned space flight to Mars. The so-called "Mars Underground" has held several conferences to discuss ideas for the mission, which, members of this loose network of enthusiasts say, could be done for as little as \$17,000 million, and largely with



technology already available or being developed for NASA's space station.

The National Space Institute, a group of space buffs that sponsored two of the conferences, has even taken to distributing "Mars Underground" lapel buttons to be "quickly flashed" at first sight of the NASA administrator, senators and congressmen.

The rise of the Mars underground has highlighted a growing rift between those who favour continued unmanned exploration of the planets — mainly planetary scientists concerned about getting the most data per dollar — and those pressing for a project that would rekindle the "thrill of exploration" that was the main dividend of the Apollo flights to the Moon. Carl Sagan has recently been trying to bridge the gap, conceding that a manned mission is not justifiable on a scientific basis alone, but urging it nonetheless in the form of a joint US-Soviet venture "on behalf of the entire human species".

NASA's current plans for Mars are limited to the unmanned Mars Geoscience/ Climatology Orbiter, scheduled for launch in 1990. Since the 1975 Viking 1 and 2 Mars landers, which raised many questions about the Martian surface, planetary scientists have been talking about follow-up missions involving a surface rover, a remotely-piloted vehicle and a return of a surface sample to the Earth.

To the extent that NASA is giving official attention to a manned Mars flight, it is in the context of advanced planning for the space station. Jesco von Puttkamer, NASA's programme manager for long-range studies — an office sometimes derisively described as NASA's division of daydreaming — says that a Mars flight is just one of five general categories of projects that an advanced space station could make possible; the others are low-

earth-orbit instruments, commercial development of geosynchronous orbit, Solar System exploration with unmanned probes and establishment of a permanent lunar base. A space station would be a necessary jumping-off point for a Mars mission because of the sheer mass involved. Puttkamer says that a bare minimum weight for a Mars craft would be 2 million pounds; the huge Saturn V rocket built to launch Apollo to the Moon had a capacity of only 250,000 pounds. The space shuttle carries 65,000 pounds. Thus, assembly in Earth orbit is a necessity.

Puttkamer estimates the cost of the first Mars flight at \$50,000–65,000 million; even that figure assumes a larger shuttle and an advanced space station would already be available. And because the Earth and Mars are in a favourable orientation for a transit only every two years, a one-shot flight makes little sense. Nobody knows how much a continuing programme of Mars flights, including establishment of a Mars

base, would eventually cost.

The more enthusiastic enthusiasts, such as the Planetary Society, put the costs much lower (\$17,000 million) and argue that new technology (such as aerobraking — using the atmospheres of Mars and the Earth to slow the craft down, instead of retrorockets) could cut fuel demand — and thus weight — considerably.

Some have also tried to raise the familiar Red Menace (which was good enough to get a man to the Moon) as a reason for pressing ahead. But according to a Congressional Research Service study by Marcia Smith, the Soviet capability argues against a serious effort on their part to put a man on Mars. The Soviets are apparently planning to send an unmanned probe to Mars's moon Phobos in 1986. But despite continued interest in Mars and a growing expertise in long-duration space flight, Soviet space hardware continues to be plagued with low reliability. Only one of the eight Soviet Mars probes has been an unequivocal success; the rest have missed the planet, crashed or failed to return data. And the Soviets have yet to try putting a man on the Moon. **Stephen Budiansky**

Biotechnology

Agrigenetics forced to merge

Washington

THE acquisition earlier this year of Agrigenetics by Lubrizol Corporation may herald a new trend in the biotechnology industry. Things have come a long way since the heady days of summer 1983, when it seemed that any company offering expertise in the new technology could do no wrong in the eyes of investors. By early 1984, public offerings were getting a much cooler reception, making difficulties for some companies seeking development capital. Agrigenetics, based in Boulder, Colorado, had seemed one of the industry's brightest hopes, having an impressive array of research expertise. But a combination of high interest rates and a depressed agricultural products market, together with the decline of the high-technology securities market, eventually forced it to seek a merger with a much larger company.

Lubrizol is saying very little about its business plans, but does not intend to alter the direction of Agrigenetics' research, which may even be augmented. But there must also be some doubt whether Lubrizol, as sole owner, will want to renew the limited partnership deal it struck with Oppenheims of New York in 1981 and which expires in 1986. That programme, which supports research worth \$11 million a year in 24 universities across the country, would be missed by many.

Analysts are cautious about generalizing too much from the Agrigenetics experience, since its business strategy was unusual from the start. Under chairman and founder, David Padwa, the company acquired seed companies to form a

distribution network at the same time as expanding its research. Reliance on the agricultural products market may have increased its vulnerability with US agriculture in the doldrums. The research arm was spending \$19 million a year principally on developing new crop varieties by applying new techniques such as the microinjection of DNA into plant cells, the use of Ti plasmids and the exploitation of transposable elements. By having a distribution network already in place, the company hoped to be able to steal a march on competitors when products of the new technology eventually reached the market.

Things began to go wrong in November 1983, when the company decided to abandon a public share offering because of the deteriorating market. Looking instead at merger possibilities, Agrigenetics found Lubrizol, a manufacturer of special oils and oil additives, a likely mate. The company already owns 15 per cent of Genentech Inc., regarded by many as the Rolls-Royce of the biotechnology industry, and was seeking ways of using genetic engineering technology to produce improved vegetable oils.

Outstanding Agrigenetics shares were exchanged for 2.1 million Lubrizol shares worth \$48 million, and in addition Lubrizol took on Agrigenetics' debt of \$60 million. In the circumstances, Agrigenetics shareholders could have done much worse. Padwa resigned as chairman last month, but denies differences of opinion with the new owners over strategy, and may continue to serve occasionally as a consultant. **Tim Beardsley**

Poland

New directions for science

NEXT October, a grand "Congress of Polish Science" will be convened in Warsaw. The initiative for the Congress came from the government and party authorities, not from the scientists, most of whom seem to know little, so far, of the purpose of the proposed gathering. It seems, however, to be intended as a working meeting, not as a demonstration to the outside world that all is well with Polish science. For, according to Dr Ignacy Malecki, a leading member of the Polish Academy of Sciences, no foreign visitors will be invited. (Until his recent retirement, Dr Malecki regularly represented Polish science at the quasi-diplomatic level of ICSU and similar meetings.)

This does not mean that Polish science is going isolationist but rather that the congress, which, Dr Malecki hinted, will include a major session on technology and innovation, represents a determined effort to put science on a new path. For the present system of research support, based on a complex hierarchy of "problems" from the "governmental" down to the "branch" level, is in disarray and is expected to be phased out at the end of this year, when the current five-year plan comes to an end. Last December, the former Ministry of Science, Higher Education and Technology lost its responsibility for technological research and innovation, which was handed over to a new State Committee for Technical Progress that will be specially empowered to direct research funding to those areas likely to provide a short-term economic pay-off.

Even more significantly, science funding for the next five-year plan will give priority to fields such as computer science, electronics and biotechnology that were judged to be of particular contemporary significance by the recent Comecon summit. The support will be generous by Polish standards; in the case of biotechnology a sum of 15,000 million zloty (£100 million) has been mentioned, although some of this, at least, must be made available in hard currency, if even these leading areas are not to suffer from the endemic problem of contemporary Polish science — a lack of funds to purchase from the West journals, reagents and spare parts and replacements for ageing equipment.

These announcements have been received by the Polish scientific community with mixed feelings. The old system of funding by "problems" was universally unpopular. It caused considerable administrative difficulties whenever an institute wanted to buy some standard equipment, say a microscope, to be used by several teams working on different "problems", and ate into the time of the unfortunate members and employees of the Academy of Science who had to coordinate the efforts of scientists working on the same "problems" in insti-

tutes scattered all over Poland. The large sector of "branch" research institutes, belonging neither to the academy nor the universities, is in considerable confusion, and few would question, in principle, the need for new legislation to cover that sector. Even the increased emphasis on practical applications is viewed by many as an unfortunate necessity of Poland's continuing economic crisis.

What the scientists find less acceptable, however, is that decision-making on priority investment in Polish science has, effectively, been transferred from Warsaw to Moscow. They fear, too, that upgrading research with practical applications will be used to justify cutbacks in other sectors, so as to cover a purge of political undesirables, terminating their research contracts on the grounds that no more funds are available. A painful example to scientists and scholars of every discipline is the fate of the Institute of Nuclear Research (IBJ) which in the winter of 1982-83 was dissolved and refounded as three institutes to weed out pro-Solidarity personnel without formally contravening the labour laws. If this could happen to IBJ, they argue, at a time when the government had just relaunched its nuclear energy programme, what are the prospects for an astrophysicist, a logician or a sociologist who falls foul of the system?

Not surprisingly, the trauma of martial law still haunts the scientific community. Formally, of course, all was forgiven and forgotten under the amnesties of 1983 and 1984. Interned and imprisoned scientists have been reinstated and the situation at IBJ officially explained as voluntary redundancies, premature retirements and the like. In fact, some of those reinstated under the amnesties have since been dismissed or eased out of their jobs (anecdotal evidence suggests that this process has been stepped up since the beginning of this year), while some formally convicted of offences against the martial law regulations have not been able to return to professional employment.

There seems to have been little consistency; of the two physicists on trial for anti-state activities when the 1984 amnesty put an end to the case, Dr Henryk Wujec has been allowed to return to his old place of employment, the Standards Office (through transferred to a different department), while Dr Zbigniew Romaszewski is unable to find a scientific post at all. A mathematics student convicted of distributing leaflets under martial law has found that, after graduation, he cannot obtain a job, whereas a class-mate similarly convicted has found no difficulty.

In some cases, exemplary punishment seems to have been inflicted almost at random: Dr Zbigniew Grabowski, a physical chemist, for example, had his election to

the Academy of Sciences vetoed by the government, although his only "offence", like that of hundreds of his colleagues, was signing a petition against the imposition of martial law.

A particularly unpleasant penalty, which has revived the fears of the whole scientific community, was that inflicted on Dr Zofia Kuratowska, who, during martial law, had headed the medical service for the internment camps. Dr Kuratowska is employed by the Advanced Studies Centre of the Polish Academy of Medicine, and runs advanced training and updating courses for doctors and medical graduates from all over Poland.

In 1983, just as martial law was coming to an end, she says, she was informed by the security police that they intended to put an end to her professional career by closing down her clinic at the Barska Street a hospital in Warsaw. In spite of petitions from doctors, patients, nurses at the general public and last-minute appeal to the Council of State, the clinic was closed on 31 December 1984 (ostensibly because the wards were needed for other purposes), leaving 500 haematological and geriatric patients without proper medical cover, and interrupting the postgraduate courses.

Dr Kuratowska has found temporary accommodation for the courses (at least until the end of the year) at the partly-built Oncological Centre Ursynow, on the edge of Warsaw. She has no in-patient facilities there, however, and, she reports, five patients have already died as a result of the closure.

Such incidents, sporadic as they are, are sufficient to make the Polish scientific community apprehensive for its future. Already the autonomy of the universities, won in the Solidarity period, is under attack and the proposed new law on the Academy of Sciences, which was still being drafted when martial law intervened, has been quietly shelved.

A major innovation of that law would have been to make the academic secretary of the academy responsible to his fellow members and not, as at present, to the prime minister. How significant such a change would have been may be illustrated by the Gradbowski case: not only was his election to the academy vetoed, but the very fact of the election was stricken from the academy records.

To an increasing number of Polish scholars, the prospect of a purge is now only a matter of "when" and "how severe". At present, scientists and academics unable to find professional work can try to continue their theoretical research privately. An unofficial Social Committee for Learning administers a few bursaries for such scholars, at parity with the official pay scales for academy researchers (about two-thirds of the national average salary). A scheme that can help a couple of dozen distressed scholars however, is clearly not equipped to cope with the victims of a major purge.

Vera Rich

French science

Good times are here again

THE French socialist party, which forms the present government in France, took a knock at the polls last weekend when right-wing parties scored 58 per cent of the vote in local elections. Of course, the French economy took its dive later than others in the West, and the electorate blames it on the new government. They may be right. But President Mitterrand's administration does have at least one feather in its cap — science — and it was seen to be waving it vigorously last week before the elections.

According to a communiqué from the council of ministers last week, French research and development spending has leapt more than a fifth in real terms since 1980, from 1.85 per cent of gross national product in 1980 to 2.25 per cent this year. Basic science may not have done so well (against a rising dollar) but "France has thus begun to regain its position among its principal partners and foreign competitors", the communiqué claimed. The benefits would come later, the investment "furnishing a solid base or the second step in the politics of industrial renovation".

The trouble is that the first step has been restructuring and unemployment. The electorate, unfortunately for the government, observes decline now more clearly than growth later. Nevertheless, the government has again pinned its colours to scientific growth at an important moment, when the "law for science", the creation of the first socialist science minister, Jean-Pierre Chevènement, nears the end of its term.

To replace it, Hubert Curien, the present science minister, promises a new law to be presented to the National Assembly in the spring, while the council of ministers is announcing that "research and development are, in effect, the keys to the modernization of our country". Auspicious times for French science appear to be here again.

Yet there are problems. Curien has been making an assessment of the successes and failures of science policy over the past three years, and describes it as "incontestably positive". But industry has been slow to invest in research; the new labour rights of researchers (which are supposed to increase mobility) have only just come into force, and many basic research laboratories are suffering from under-equipment and too few hard dollars for materials and apparatus. French computing power is also way behind the competitors, despite a decision last year to allow laboratories — after ten years' moratorium — to buy foreign machines. Much cash was also wasted on ill-conceived national technology programmes.

The necessary structural reforms have now been accomplished, says Curien; the great revolution has been the acceptance by

a largely left-wing university system of the need to cooperate with industry and by industry of universities as partners. The stage is set for real development, Curien would claim. "The decline of Europe in relation to the United States and Japan is not written in history", he wrote last week in the newspaper *Le Monde*. And France must develop with Europe: "Is science not one of the best cements for a still fragile but indispensable European cooperation?", he asks.

In France, a commission has been set up to study the proposed law for science. But already the council of ministers has

Italian biotechnology

Planning to catch up rivals

Rome

A NEW centre for biotechnology research and development is being founded at Pomezia, 45 km south of Rome, by Menarini SaS, the Florence-based pharmaceutical company. Dr Alberto Aleotti, president of Menarini, says the company is investing 28,000 million lire in the new biotechnology centre.

The first goal of the centre will be the manufacture of TPA (tissue plasminogen activator) and its derivatives for human pharmaceutical purposes, using recombinant DNA technology developed by Creative Biomolecules, a California corporation founded in 1982 by the 36-year-old Italian, Roberto Crea, and Charles M. Cohen.

The agreement between Menarini and Creative Biomolecules is aimed at the production of an economic drug for the international market. The Pomezia team will include researchers from Italy, the United States and Britain. The first TPA for chemical and biological evaluation is expected in the summer. Crea, acknowledging that his project is not the first in the field — Genetech first cloned TRA in 1982 — says its objectives are superior.

Inaugurating the Menarini centre (and the toxicology research facility on the same site), Italian minister of research Luigi Granelli pointed to several encouraging signs of development in Italian biotechnology by way of international cooperation. The international genetic engineering centre of the United Nations Industrial Development Organization, to be based at Trieste, is one of these. But Granelli added that developments in this field in Europe as a whole are still not a sufficient counter to developments in Japan and the United States, perhaps because governments see their interests as stronger than supra national ventures.

Italian biotechnology is in part held back by the lack of venture capital, with the result that most of the companies involved

promised scientists several benefits:

- An increase in cash available for laboratory equipment.
- A long-term policy for training and employment in research and development.
- The establishment of research more widely in industry.
- Continuance of the "great programmes of technological development" such as space, aeronautics, energy and the oceans.
- At the same time, reinforcement of the assessment of these programmes and other action so that corrections can be applied when things go wrong.
- Improvement of tax and other financial incentives to industry and to research.
- Continuance of the "strong priority" given to research and development.

Robert Walgate

are either well established in some related field such as pharmaceuticals or are public enterprises. The companies with declared interests include Farmitalia-Carlo Erba (Montedison group, now under the umbrella of Erbamont), Lepetit and Serono. Sorin Biomedica (wholly owned by Fiat), Farmitalia and various companies of the ENI group have declared an interest in protein chemistry; Sclavo, an ENI company, plans to produce beta-interferon. Biotechnology in agriculture is quite advanced at ENEA, the public corporation for nuclear and alternative sources of energy, and Assoreni (ENI group) has interests in agricultural biotechnology.

University researchers have been drawn into biotechnology in various ways. Thus the multinational Roche facility at Milan has an agreement with the Pharmacology Institute of the University of Milan, headed by Professor Rodolfo Paoletti, for applications to molecular neurobiology. The Italian Society for Biotechnology (SIB) has signed a convention with IASM, the public sponsor of development in the south of Italy, and with FINAM, a financial holding for agriculture, to set up a centre for biotechnology transfer in the south.

The first objective of the centre will be to evaluate the degree of innovation in small and medium-size industries so that by the end of 1985 agricultural experiments on a pilot-plant scale may be started.

Projects sponsored by the Consiglio Nazionale delle Ricerche (CNR) have modest achievements to report. While the CNR genetic engineering programme, launched in 1982 with 20,000 million lire has so far recruited only a few participants among Italian industries, a 1980 project (due to finish this year) on fine chemicals under the direction of Professor Luciano Caglioti has worked with 500 different entities. So far, the project has published 1,800 scientific papers and produced 150 patents, some of which have already gone into production.

Paola de Paoli

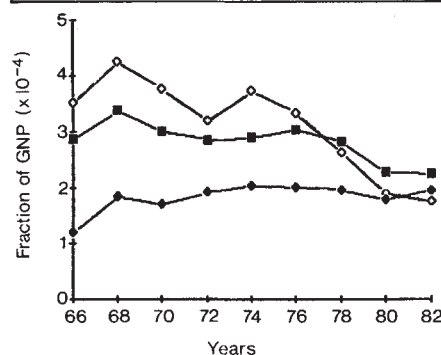
CERN

Reduced budget in prospect

Geneva

BRITISH officials are beginning to sound out the 13 member states of CERN, the European Organisation for Nuclear Research near Geneva, to see if they would countenance a substantial reduction in the CERN budget (which amounts to some £250 million a year) while a fourteenth potential member, Portugal, has applied to join. Could Portugal pay for the reduction Britain requires? Certainly not, but the eagerness of Portugal and the dog-in-the-manger attitude of Britain make a sharp contrast seen from CERN.

Even Britain's backing of salary increases at CERN (*Nature* 7 February, p.423) could be a ploy in the long run of reducing salaries; negotiations begin this year for a major two-year review of the



The decline of Britain's spending on high-energy physics (open diamonds) as a fraction of gross national product, compared with that of West Germany, France, Italy and the United Kingdom jointly (full squares) and Belgium, the Netherlands, Sweden and Switzerland (full diamonds). Salaries are included.

By 1982, UK spending was already below that of the latter "medium size" members of CERN.

CERN salary structure. Britain, it is said, did not wish to begin on the wrong foot from the start of these negotiations (which Britain will chair) by backing a sudden and unprepared French move to cut pay.

Thus Britain's head-counting looks ominous — particularly in the light of a committee chaired by molecular biologist Sir John Kendrew which will report soon on the value of Britain remaining a member of CERN. Sir John may praise the performance of CERN — it is, after all, at an all-time high in its history and even leading the world — but he may also criticize costs.

Britain's real spending on high-energy physics has been declining rapidly (see Figure), but European investment remains a third above that of the United States when measured as a fraction of gross national product. CERN argues that this reflects the superior quality of CERN accelerators, and that without this quality Europe would not be in such a commanding position. Fermilab in the United States is three years behind CERN in creating the

antiproton-proton collisions that at CERN created the W and Z particles and now hint at supersymmetry. But British accountants may find the benefits imponderable.

CERN, however, says that their next big accelerator (the large electron-positron collider, LEP) is being built on constant budgets, so that cuts are already being made throughout CERN to pay for LEP's construction. The director-general, Professor Herwig Schopper, is cutting staff, and accepts a Thatcherite hypothesis that every organization has some fat to lose. "But we're now cutting to the bone", says research director Ian Butterworth.

The bone is research. Over the past five years CERN has run double the number of research hours of its most direct competitor, Fermilab, says CERN technical director Giorgio Brianti. "We run a good deal — 7,000 hours a year, more than most nuclear power stations." But research after all is what investment in CERN is for.

Yet to cut running time, and hence electricity bills, might be the only real cut that CERN could now make. Already 70 per cent of the capital spending on LEP is committed. And salaries (half the CERN bill), while high by national standards, are low by international ones.

If running time were cut, physics would slow down, and competition from the United States would be more threatening.

Britain's possible complete departure from CERN is greeted with horror and disbelief in Geneva. CERN claims to have no contingency plans for the loss of Bri-

tain's 17 per cent contribution, or its physicists, though there would be a year or two to act (the constitution requires withdrawal on the January after the January following announcement of withdrawal).

Carlo Rubbia, the Italian discoverer of the W and Z particles, says Britain should go if it wants to, and not hang about like the character in Verdi's opera *Aida* who sings "I'm going, I'm going" for five acts but never leaves. Bob Brown, a British physicist in one of the four giant experiments to be mounted on LEP, says Britain's leaving "is too awful to contemplate". His experiment, OPAL, in which Britain has a major contribution (the provision of a lead-glass scintillator) "would collapse" if Britain left. Another LEP experiment, DELPHI, might be threatened.

Others say Britain just could not spend effectively the £50 million that the Science and Engineering Research Council would be left with if Britain left CERN; and that the research students presently staying in Britain to do high-energy physics (80 per cent of them with first class degrees) would simply go to the United States, weakening British science. In the longer run, non-members do participate in CERN experiments, but they make triple the normal contribution to apparatus, and Britain's problem would not thereby be solved.

"It would be a disaster" if Britain pulled out, said Schopper last week. "Would we throw out the British physicists? I cannot see that. But what would the other states say?". There would, in the end, be a limit to how long they would be willing to bail out a defaulter. **Robert Walgate**

No home yet for radiation source

THE European Synchrotron Source (ESRS), the 5-GeV light source that France and West Germany agreed should be built in France (in exchange for a supersonic wind tunnel to be built in Germany), is up for discussion yet again.

Now it is the turn of the Levaux Commission, the supposedly intergovernmental body set up by the European Science Foundation with the original aim of deciding the location of ESRS. France and West Germany effectively spurned the commission and decided among themselves (with British assistance) to put ESRS in France, hoping that the smaller states on the commission would agree.

But they did not; the situation was further complicated when the French government switched choices from Strasbourg (close to the German border) to Grenoble (in the French Alps), probably for electoral reasons. West German officials had always favoured Strasbourg, and were severely put out (though philosophical) when the French government changed its position.

Thus, when the Levaux Commission meets this week, French representatives will

be all but isolated. France needs cash support to establish ESRS, and it is conceivable that strong pressure will be put on France to change its mind yet again. The regional elections feared by the French government, which could have displaced Louis Mermaz, the socialist president of the National Assembly, from his seat on the Grenoble regional assembly and perhaps precipitated a national election, are taking place last Sunday and next, so the internal political pressure will soon have changed. Not a stone has yet been turned for ESRS.

So could ESRS go to Strasbourg after all? Not if Denmark or Italy have their way, as Denmark still champions Risø and Italy Trieste. Scientific site visits to both places have taken place since France proclaimed the site would be Grenoble, and Italy continues to contest very loudly on behalf of Trieste. Last week, however, Italian physicists meeting in Trieste were talking of a compromise — the building of a 1.5 GeV synchrotron radiation source to "complement" the X-radiation from the 5-GeV ESRS in the lower wavelengths (soft X-rays and ultraviolet). **Robert Walgate**

Imunovir defended

SIR — In a letter published on 31 January¹, Dimitri Viza makes a number of assertions about Imunovir (inosine pranobex) and the "aggressive publicity campaign" which he suggests has been mounted to promote it. M. Viza's intention seems to be to criticize not just the product but also the journals and newspapers (specifically *Nature*, the *Sunday Times* and the *Financial Times*) which published articles on Imunovir (which is spelt with one m). In doing so he has made some errors, which I would like to correct.

First, M. Viza states that "... this drug is already better known from its promotion than its results". More than 300 papers have appeared on Imunovir, including many in journals such as *Lancet*, *International Journal of Immunopharmacology* and *Antimicrobial Agents and Chemotherapy*. As an example of this "promotion", M. Viza states "... US drug-store bookstalls hyped the drug with a paperback which claimed a cure for herpes ...". Although the author did not support this claim with any scientific publications, the book claimed him to be 'an authority on herpes'. The book in question, *Herpes: Cause and Control*², is an independent text on herpes by Dr William H. Wickett, former director of Student Health Services at California State University, Fullerton. The book discusses a wide range of preparations used to treat herpes, including acyclovir, idoxuridine and Imunovir. However, the latter is at all times referred to as "the test drug": not once is any generic or brand name used, and Dr Wickett states that this is at the request of the manufacturers. It is difficult to see how a book can "hype" a drug without once referring to it by name. Moreover, the book does not claim a cure for herpes. I quote Dr Wickett: "We found that those patients taking the test drug were cured of their Herpes bout in nearly 60% of the cases at the end of one week. Those taking the placebo were cured, by the same criteria, in about 14% of cases." Assuming that M. Viza is a doctor, he should know that herpes simplex is a recurrent disease and that curing a "bout" is not at all the same thing as curing the disease itself.

Neither the US discoverers of Imunovir, Newport Pharmaceuticals, nor Edwin Burgess Limited which distributes the product in the United Kingdom, has ever claimed that it can cure herpes. To quote the *Financial Times* article³, also criticized by M. Viza, "[The company] is quick to point out, however, that the product is not a cure for viruses like herpes, only a moderator of the disease".

This brings me to another assertion, that if Imunovir is effective in herpes, the number of patients in France, Germany, Italy and Spain, where the drug has been available for some years, would have decreased. Imunovir certainly appears to

reduce the severity⁴⁻⁸ and frequency^{9,10} of recurrent attacks of herpes simplex, but as it is not held up to be a cure, the number of patients with herpes can hardly be expected to decrease. In addition, the number of cases is probably related more to increasingly relaxed sexual attitudes than to available therapy.

To move on to the criticism of the article in the *Sunday Times*¹¹, it seems to be the headline that has caused offence: "Pill for AIDS — but it may come too late". Headlines are written not by authors of articles, but by sub-editors. In fact the *Sunday Times* article referred to work in "pre-AIDS" patients in New York, the results of which were presented to an audience of genitourinary specialists in London and are at present awaiting publication. The work was sufficiently encouraging for similar trials to be set up both in London and the United States to see whether the findings can be confirmed. These are now under way. The statement in the *Financial Times*³ — "Imunovir may be able to prevent AIDS" — also refers to the New York study. M. Viza takes objection to another statement from that newspaper which suggests that Imunovir shows promising potential in a variety of diseases. I would like to draw his attention to the words of the jury which awarded Imunovir the "Prix Galien" in 1982, an award established in France to recognize major thera-

peutic innovation. (Other products honoured by the award include rifampicin in 1970 and cimetidine in 1979.) The distinguished professional jury stated that Imunovir "has transformed the basis of immune therapy and opens new hopes in the field of medicine as important and diverse as viral diseases, autoimmune disease, allergy, parasitology and cancer".

M. Viza is right when he says that responsible lay publications should not give rise to false hopes about potential "break-throughs", and I believe that we would all be glad to see an end to sensational headlines which bear little relationship to the article, and to the "wonder drug" type of story in general. I dispute the idea that the articles to which M. Viza refers, including the one in *Nature*¹², are anything but well-balanced, responsible pieces of reporting based on the scientific evidence available.

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Status of IVF embryos

SIR — An unfertilized egg is both human and alive, being a detached bit of its mother's body; but it cannot develop further without something positive being done to it from outside, namely fertilization. Otherwise it must inevitably die and, since nobody would claim that it therefore has a right to be fertilized, some would argue that if the "right to life" depends upon being human and alive, fertilization should make no real difference in this respect. But there is of course a fundamental difference, because when the egg fuses with the sperm a new living human organism comes into being, biologically as distinct from both of its parents as it will ever be, and none the less so for the possibility that it may still be able to give rise to twins. This newly created organism has within itself the potential to complete its development as a human person, which it will normally do unless it dies of its own accord, or is killed by a procured abortion. Whether or not at such an early stage it is to be regarded as a "human being", or only potentially one, it is hard to see what difference this should make to its rights to be allowed to realize its full potential without being deliberately destroyed.

There is however an important difference between the potential for further devel-

opment possessed by an egg fertilized normally inside its mother, and an embryo produced artificially by *in vitro* fertilization (IVF) outside her body. This is that whereas the embryo *in utero* has of itself the full potential to complete its development without any further intervention from outside, that is not so with IVF, when it requires another positive act in putting it back into a woman's uterus.

So far as completing its development is concerned, therefore, the IVF embryo can be compared with an unfertilized egg, since neither can do this without some positive intervention from outside. It is this, rather than the small size and lack of any visible signs of humanity, that may justify the use of IVF embryos for experimental work expected to result in death, which would anyway be unavoidable whether or not the experiments had been performed. The ethical problems that arise when embryos *in utero* are to be intentionally aborted are certainly quite different from those involved with experimentation *in vitro*, since even from the earliest stages they would otherwise have had a chance of completing their normal development.

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Unborn Children (Protection) Bill

from H. John Evans and Anne McLaren

If the bill recently presented in the British Parliament were to become law, many promising avenues of research would be blocked.

MUCH to the dismay of most informed scientists, infertile couples and parents at risk of producing children with severe inherited genetic defects, Mr Enoch Powell's private member's bill effectively to ban studies on human embryos created by *in vitro* fertilization (IVF) won a majority when given its second reading in the British House of Commons, on 22 February.

The bill seeks to "render it unlawful for the human embryo created by *in vitro* fertilization to be used as the subject of an experiment or indeed, in any other purpose except to enable a woman to bear a child" (Hansard, 15 February, 1985). The bill does not seek to interfere with "procedures that are in use at present for enabling women who would not otherwise be able to do so, to bear children", but will, despite Mr Powell's comments to the contrary, effectively block any attempt to improve these, at present, rather inefficient procedures. The present success rate for human embryo transfer is of the order of 10 per cent. There is, however, no obvious reason why medical science should not be able to increase the efficiency of IVF and embryo transfer to achieve a success rate of at least 50 per cent. This will not be possible if this bill becomes law.

The passage of the bill would deny many thousands of couples in Britain (and 1 in 10 couples are infertile) the means to produce a family of their own, but this is not the only reason why a recent leading article in this journal (*Nature* 7 February, p. 417) described this bill as "home-made and half-baked" and a "blunderbuss" of a bill. Unfortunately, the bill will also prevent attempts to circumvent the production and transfer of severely genetically abnormal embryos, a possibility that is almost within the grasp of medical science, and which is ignored or rejected by proponents of the bill.

The Pro-Life group have lobbied all members of the British Parliament and organized petitions in support of the bill. Last month, Professor Jerome Lejeune was imported from Paris to address the Parliamentary Pro-Life group, and his views on curing inherited genetic disease¹ have been frequently quoted, misquoted and embellished, in support of the group's aims.

It is claimed that severely mentally retarded children with the fragile-X syndrome show improved mental development following treatment with

folic acid; that azacytidine treatment of patients with β -thalassaemia may provide an effective therapy for this, and indeed other, inherited diseases; that vitamin therapy to the mother will drastically diminish the risk of spina bifida, and that these examples illustrate that experiments on human embryos are not required. They do no such thing. In fact, they are something of a red-herring, but before dismissing them we should at least look at the facts.

Therapy

The fragile-X syndrome afflicts some 1 in 1,000 males with mental retardation². One diagnostic feature is the presence of a constricted and fragile region at the end of the X chromosome which is visible in a proportion of cells cultured in a medium deficient in folate. Adding folate prevents the cytological expression of this site³. This fragile site and the locus causing this form of mental retardation are closely linked, but it does not follow that they are one and the same locus. Indeed, the fragile site is not always readily demonstrable in males apparently afflicted with this form of mental retardation^{4,5}, while some mentally normal males possess a fragile X chromosome⁶.

Folate deprivation also reveals reversible fragile sites on many other chromosomes⁷, and they are not associated with mental retardation. The use of folic acid as a treatment for X-linked mental retardation has nevertheless been attempted in a number of small, and often uncontrolled, trials. Subjective "improvements" in the behaviour of some, but not other, folate-treated children have been reported, but with borderline⁸ or no^{9,10} changes in IQ. The results of these trials are unimpressive.

The proposed treatment for β -thalassaemia raises other questions. 5-Azacytidine (5-AZ) may be incorporated into DNA in place of thymine, but, unlike thymine, it cannot be methylated. Thus, in the presence of 5-AZ, genes whose functions are normally suppressed by methylation may become active¹¹. In man, the genes coding for fetal γ -globin become methylated and turned off ("inactivated") after birth and their role in haemoglobin formation in the adult is taken over by the β -globin genes. So in patients with β -thalassaemia, where the genetic defect results in the absence of adult β -globin, one approach for therapy would be to maintain, or restore, the activity of the

fetal γ -globin genes to compensate for the absence of functioning adult genes.

In 1982, Ley *et al.* reported¹² that a seven-day 5-AZ treatment of an adult patient with β -thalassaemia resulted in a temporary increase in the level of a fetal γ -globin. 5-AZ is, however, a very dangerous drug; it is likely to be carcinogenic and it also activates latent viruses. Its use would almost certainly be regarded as unethical in the United Kingdom. Although the rationale behind this possible therapy is clear, the longer-term consequences are not. Certainly more than the γ -globin DNA sequences would be expected to be "turned on" in proliferating cells and this kind of treatment could have bizarre, even lethal, consequences.

The third example quoted by the Pro-Life lobby is different in that it relates to a treatment of the mother who bears the fetus and is a preventative therapy. The possible role of vitamin supplementation in reducing the incidence of neural tube defects has been rehearsed in these columns before and will not be repeated. We might note, however, that the Medical Research Council has set up a "multicentre vitamin supplementation trial" to which the Pro-Life lobby has objected.

Understanding

Inherited defects contribute substantially to human ill health and considerable efforts are being expended in attempts to understand their nature and cause. This work very largely involves the use of cultured human somatic cells and its fruits could, in practice, be applied in various ways.

Defining and understanding the nature of the defect can lead to rational attempts to alleviate the disease, by supplementation (*à la* Lejeune) or by circumvention. Attempts at alleviation could, in theory, be undertaken at any stage in development. The widely publicized approach of somatic cell gene therapy in the late fetus or adult, using retroviral or plasmid-based expression vectors¹³, and the recombinant DNA research that underpins it, have been the subject of much emotional debate¹⁴.

Yet these developments are some way removed from practicality and, it should be emphasized, will be applicable to only a limited number of diseases. Moreover, they are concerned with attempted amelioration of the disease and not with its prevention. And they involve manipulation of the patient, not of the embryo.

Lejeune has made the point that research aimed at alleviating the symptoms of mental defects, as in Down's syndrome, cannot be performed on early human embryos, because at that stage the central nervous system has not even begun to develop. Similarly, he claims that research on the alleviation of muscular dystrophy cannot be carried out before the muscles are present. These are valid points, because Lejeune is concerned only with therapy. What Lejeune omits to say is that research on the detection and prevention of these genetic defects, and studies designed to increase our understanding of their aetiology, not only *can* be done on the pre-implantation conceptus (early embryo), since the defective genes or chromosomes are present throughout development, but in some instances can *only* be done at this early stage.

Unfortunately, some members of the Pro-Life lobby confuse the attempted treatments of patients with an inherited disease using genetic manipulations of the somatic cell, which may well be a desirable approach, with genetic manipulation of the embryo, which is not only clearly undesirable, but also unnecessary. With severe single-gene inherited disease, 50 per cent of the germ cells of carriers are normal. For an autosomal recessive disorder such as cystic fibrosis, 3 of 4 fertilized eggs produced by two carrier parents (around 1 in every 22 people in the United Kingdom are carriers of this abnormal gene) will result in normal children; for a carrier female of an X-linked recessive gene, as in Duchenne muscular dystrophy, all embryos destined to be female and one-half of those destined to be male (or 3 of 4) will be normal. A fertile carrier of a gene for an autosomal dominant disease such as Huntington's chorea will transmit that gene to one-half of his or her conceptuses. In all cases a proportion of the embryos will result in normal children. Provided we can detect which is normal and which abnormal, there is no need for genetic manipulation of the embryo.

Therefore, it is evident that if we could monitor for the presence of an abnormal gene of chromosomal constitution in an early embryo, then by the use of IVF and embryo transfer it would be possible to ensure that only normal embryos are transferred back to the mother in those families that are carriers of serious genetic disease. In the same way, infertile couples who partake of IVF and embryo transfer or those that are at risk of transmitting a chromosome anomaly, could be assured that the transferred embryos are

chromosomally normal. (The first published announcement of the birth of a chromosomally abnormal child, a Down's syndrome baby, conceived by IVF and embryo transfer, has recently been reported¹⁵.) Is it possible to carry out such monitoring? At present, the answer is no. But the means to undertake monitoring are just around the corner, and would require further studies which would become outlawed under Mr Powell's bill.

The chromosome constitution of an early human embryo can be established from a cytogenetic analysis of but a few cells, and such studies have already revealed a high incidence of chromosomal abnormality in human embryos¹⁶. The possibility exists of determining the chromosome constitution of an 8-celled or 16-celled conceptus by removing one or two of its cells and culturing them for chromosome analysis. The conceptus would in the meantime be frozen and afterwards thawed out and transferred back into the woman only if the cells were shown to be normal. (The cells of the early conceptus are multipotential and it has already been shown that a frozen 8-celled human conceptus which has lost some of its cells can give rise to a normal fetus¹⁷.)

The same approach could be used to detect the presence of specific abnormal genes. More and more DNA probes for genes responsible for severe genetic disease in man are becoming available, and further advances in technique will enable such genes to be detected in small cell samples. The means of preventing the transmission of certain severe inherited diseases (with, incidentally, a concomitant reduction in the requirement of abortions in the "at-risk" families) is therefore almost to hand, but their development and implementation will not be possible if the bill succeeds.

Infertility

It is already evident that the very high rate of chromosomal non-disjunction in our own species is responsible not only for many of the genetic defects at birth, but also for much infertility and early spontaneous abortion. We know nothing of its causes, but we do know that much of it arises during oocyte maturation, or during the fertilization process or the early cleavage divisions. *In vitro* studies of these stages of development could therefore throw light on the aetiology of chromosomal disorders.

It is in the field of infertility that research on human oocytes fertilized *in vitro* is most urgently needed and will have the most immediate impact. Already there are hundreds of couples in Britain who owe

their babies to such research. The current inefficiency of IVF and embryo transfer can be rectified only if systematic studies of the variables involved can proceed. For instance, the nutritional conditions provided for the fertilized egg *in vitro* are almost certainly not optimal. The IVF conceptus has been shown to secrete chorionic gonadotropin¹⁸, a hormone thought to be crucial for the success of implantation. Conditions of IVF that gave a conceptus secreting low levels of the hormone might well be suboptimal from the point of view of producing a pregnancy.

Male infertility is almost as common as female infertility, but is less tractable to treatment. IVF has proved useful in some cases, since fertilization *in vitro* can be achieved with relatively small numbers of sperm, but the techniques are primitive and more research into the fertilization process itself is needed.

Contraception

A further area in which IVF is already contributing is in the development of new contraceptives. Present contraceptive pills have some undesirable side effects, and attempts to develop a "male pill" have been fraught with problems. The ideal hormonal contraceptive would affect only the oocyte or the sperm and such compounds are now being developed. However, before they could be considered for use it is essential to know if they carry a risk of abnormal fertilization — this can only be ascertained through IVF using human gametes.

In an issue as important and as emotional as this, it is essential that our representatives in Parliament understand the consequences of the proposed legislation. Mr Enoch Powell's bill would make it a criminal offence for any human oocytes to be fertilized *in vitro* except for purposes of "embryo insertion". The endeavours of doctors and scientists to use IVF studies to reduce the numbers of spontaneous abortions and children born with genetic defects, to reduce the numbers of abortions induced on genetic grounds, to alleviate infertility and, by improving contraceptive measures, to reduce the rate of "social" abortions and the numbers of unwanted children — all these would cease. To call such a bill the "Unborn Children (Protection) Bill" seems a prime example of Orwell's Newspeak. □

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Is gravitational energy real?

The long-recognized problem of Einstein's theory of gravitation, that gravitational energy is not unambiguously defined, may be unavoidable. But people keep trying.

BETWEEN Newton's time and the beginning of the twentieth century, most people were broadly speaking content with the notion of the potential energy of an object in a gravitational field. Following the Galileo legend, one had merely to drop a stone from a height to see its potential energy converted into kinetic energy. By the nineteenth century, the interplay between the two had become a way of solving practical problems, most elegantly by means of hamiltonian mechanics, but more crudely (as with a falling stone) by equating decreases of potential energy with concomitant increases of kinetic energy. But Einstein changed all that.

It is paradoxical that the best theory of gravitation yet has thrown gravitational potential energy into a confusing limbo. R. Penrose, in a recent paper (*Proc. R. Soc. A* **381**, 53; 1982) puts the point directly, if inelegantly, by noting that "it is perhaps ironic that energy conservation, a paradigmatic physical concept arising initially from Galileo's (1638) studies of the motion of bodies under gravity... should nevertheless have found no universally applicable formulation, within Einstein's theory, incorporating the energy of gravity itself". But people keep trying to define gravitational energy. The latest attempt of this kind, by D. Lynden-Bell and J. Katz (*Mon. Not. R. astr. Soc.* **213**, 21; 1985), is intriguing chiefly as an illustration of the difficulties.

The underlying problem with gravitational potential energy in general relativity stems from the principle of equivalence, which logically enters the theory as an assumption, and which has as one of its consequences that an observer falling freely in a gravitational field will have the illusion that the field does not exist. In other words, it is always possible to get rid of a gravitational field by the choice of a suitably accelerated frame of reference. The principle of equivalence leads to the conclusion that free-falling objects always have the same acceleration (which is Galileo's observation). But if gravitational acceleration can always be transformed away, gravitational potential energy cannot have the significance attributed to it in classical mechanics.

Einstein naturally took up the question of how the classical conservation laws would be transferred into his new formalism. One of the frustrations that quickly became apparent is that this seemed not to be unambiguously possible, at least

in the strict sense that the representation of a physical quantity should be independent of the coordinates in which this is done (or, technically, that its numerical value at any point should transform with changes of coordinates as does a tensor quantity).

The situation is really rather odd. What Einstein's general theory does is to relate the quantities describing the curvature of four-dimensional space (and which themselves are functions of the space and time coordinates) to a quantity called the energy-momentum tensor, a true physical quantity (in the sense of being a tensor and thus coordinate-free) which represents the energy and the momentum locked up both in matter and in fields other than the gravitational field, electromagnetism for example. Einstein's problem, in the years succeeding 1917, was to find some way of representing the total energy of a system — matter-energy, the energy of the electromagnetic field and of other fields as well as the energy of the gravitational field, together with the modern analogue of the old potential energy — so as then to show that this quantity is conserved.

Einstein recognized that his solution is not ideal. The best candidate for a conserved quantity representing energy is not strictly a tensor but, a construct from the quantities describing the curvature of space-time which is not a tensor in the strict sense but; a pseudo-tensor, *viz* a quantity depending on the coordinate system is Einstein's pseudo-tensor, not a quantity that exhibits on a microscopic scale the conservation of energy and of momentum.

So is there a buried scandal in general relativity? Einstein's failure (often repeated) to find a quantity representing energy and momentum whose microscopic conservation is self-evident does not imply that energy and momentum are not macroscopically conserved. The difficulty that immediately raises its head is that the verification of macroscopic energy conservation must necessarily be possible only within the framework of a particular macroscopic description of the Universe. And many model universes in which energy is not conserved, which are nevertheless inconsistent with energy conservation, have been constructed.

Are there some circumstances in which the definition of potential energy, or the energy of the gravitational field, is possible in terms of the variables that define that field? This is the path that Lynden-Bell and

Katz have followed. Beginning with an observation due to J.A. Wheeler that the problem of the unambiguous definition of potential energy may be simpler in the conditions in which spherical symmetry applies, they seek to define the gravitational field energy unambiguously in such circumstances.

The argument goes like this. In a system in which there are only gravitating masses, there must be some quantity, a function of the space-time coordinates, whose integral over any space-like surface (say that corresponding to the present time) is equal to all the energy associated with the mass, its rest-energy and momentum, and its gravitational potential energy. The equations of general relativity allow calculation of the rest-energy from the descriptors of the curvature, so that the difference between the surface integral and the mass within the surface must be the gravitational potential energy. Thus, by considering the difference between the surface integral on two nearby space-like surfaces, it should be possible to define the density of gravitational potential energy, the field energy. Lynden-Bell and Katz pin down their calculation by making an artificial cut in space-time along some space-like surface in which, without loss of generality, they can suppose that space-time is flat.

Physically, the result makes sense. The energy locked up in the gravitational field (as distinct from the mass itself) does indeed decrease with distance from massive objects. Significantly, the field around a black hole turns out to contain the whole energy of the condensed object. This fits in well with the views of those who hold that the significant consequences of black holes are external. Lynden-Bell and Katz say that their result differs from that of the "Penrose school", a reference to Penrose's elegant generalization of general relativity to accommodate particles with spin. That, according to Lynden-Bell and Katz, puts all the energy of a black hole inside it.

The implications of this calculation will depend on whether it can be generalized to universes that are neither spherically symmetrical nor static. There seems to be good reason for hoping that something can be done. But if part of the essence of general relativity is that potential energy cannot be unambiguously defined except by reference to large-scale structure, there is bound always to be a degree of arbitrariness in the way in which the concept arises.

John Maddox

Astronomy

Gas in elliptical galaxies

from A.C. Fabian

IT HAS generally been held that early-type galaxies — lenticulars and ellipticals — are devoid of gas¹. The gas lost by stars, both as the winds from red giants and as planetary nebulae, has been presumed to be blown out of the galaxies by the cumulative effect of supernova explosions. A galactic wind thus scours a galaxy clean of gas, explaining why only relatively minute quantities (and little star formation) have been detected at radio, optical or ultraviolet wavelengths. This picture, itself, is now being blown away by evidence from the *Einstein Observatory* satellite: a considerable number, and perhaps all, early-type galaxies contain large quantities of gas that are only detectable by their X-ray emission. A new compilation of the data² shows that the mass of gas in a large elliptical galaxy can be about the same as that in our own spiral Galaxy.

One of the first results³ reported from the *Einstein Observatory* was that the Virgo cluster of galaxies appeared 'spotty' in X rays. Individual normal and early-type galaxies appeared as diffuse sources of X rays. Further work⁴⁻⁶ showed that many other normal galaxies were also X-ray sources, and attention seems to have drifted away from the early-type galaxies to more exotic objects such as active galactic nuclei.

Spiral galaxies, such as our own and Andromeda (M31), are X-ray luminous because they contain populations of X-ray emitting binary star systems. Large elliptical galaxies, however, are about one hundred times more X-ray luminous than spirals, such that over 1,000 very bright X-ray binary sources would be required. That interstellar gas is a more likely candidate for the observed X-ray emission is demonstrated by the X-ray emitting 'plume' of NGC4406 (M86; ref. 3). This large elliptical galaxy is falling through the Virgo cluster at about 1500 km s⁻¹ and much of its gas is being stripped out by interaction with the extended halo around M87, to form a tail or plume in its wake. The nearby active galaxy Centaurus A also shows diffuse X-ray emission⁷ (apart from the nucleus, jet and so on) which is most likely due to a hot interstellar medium, and not binary X-ray sources. The gas in elliptical galaxies should be at X-ray temperatures, for gas lost from stars moving at speeds σ , relative to the galaxy, of a few hundred km s⁻¹ should thermalize to a temperature of between 10⁶–10⁷ K such that its sound speed is about σ .

W. Forman, C. Jones and W. Tucker have compiled² the *Einstein Observatory* X-ray data on 55 early-type galaxies, most of which show X-ray coronae, and have obtained results that are of considerable significance to studies of early-

type galaxies. Crude spectral analysis shows that the emission is consistent with that from hot gas and not with that of known classes of X-ray binaries. Thus, most optically luminous lenticular and elliptical galaxies contain hot gas; they cannot blow winds. Supernova heating, which could have ejected the gas, must be much less than previously assumed.

An important application of these hot gaseous coronae is the measurement of the masses of early-type galaxies. If the gas is observed, then its bulk motion must be highly subsonic, otherwise impossibly large sources of mass are required to maintain a detectable density. Consequently the coronae must be close to hydrostatic equilibrium and

$$\frac{dP_{\text{gas}}}{dr} = -\rho_{\text{gas}} \frac{GM(r)}{r^2}$$

Measurements of the X-ray surface brightness and temperature profiles yield the gas pressure, P_{gas} , and density, ρ_{gas} , and thus the mass as a function of radius $M(r)$. The compiled data yield total masses of about $1 - 5 \times 10^{12} M_{\odot}$ out to 100 kpc for the best studied cases. Some of these galaxies are in the Virgo cluster but most are in the weak groups and some are relatively isolated. The X-ray data imply massive dark haloes around most elliptical galaxies. Their mass-to-light ratio is around 100 in solar units.

For detectable amounts of hot gas to accumulate in galaxies the gas must be cooling at a significant rate, already discussed in the context of a small subset of the present sample⁸. Gas must be cooling and objects condensing out of the resultant cooling flow at a rate of about one solar mass

per year, so that the formation of stars in large elliptical galaxies take place at a rate not much less than it does in our own Galaxy. Their optical appearance, however, provides no indication for this. The conditions must be such that the high-mass stars that we normally associate with regions of star formation in our Galaxy are absent. The new stars in elliptical galaxies must be of very low mass, perhaps even below that for nuclear burning. We conclude that elliptical galaxies are not quite so full of old stars as previously thought, although one solar mass per year for a Hubble time does not yield a $10^{12} M_{\odot}$ galaxy. Nevertheless there are intriguing implications for the formation of galaxies and of the so-called dark matter associated with them.

We thus have an extremely powerful tool for measuring the mass profiles out to distances of 10–100 kpc and for studying the gas and energy flows in early-type galaxies. When are we going to see more data? Unfortunately, the *Einstein Observatory* re-entered the atmosphere three years ago and the telescope on the EXOSAT satellite is not sensitive enough to help out. ROSAT, to be launched in 2–3 years time, should detect many elliptical galaxies out to 100 Mpc or more and, in the 1990s, high-quality imaging X-ray spectroscopy should become routinely available for any elliptical galaxy. □

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Congenital diseases

Complementary genes for an adrenal enzyme deficiency

from Ian R. Phillips and Elizabeth A. Shephard

ONE of the most common inborn errors of metabolism, congenital adrenal hyperplasia, results from a deficiency in the adrenal cortex of the enzyme 21-hydroxylase¹, which is involved in the biosynthesis of cortisol, the principle glucocorticoid hormone of the adrenal. The disease is inherited as a recessive trait and is closely, but enigmatically, linked to the major histocompatibility complex of humans, *HLA*. Two separate reports now help explain the linkage. First White *et al.*² show that the disease is due to a defect in a gene coding for cytochrome P-450_{C21}, a pro-

tein that is specifically catalytic for the 21-hydroxylation of steroids. Second, Carroll *et al.*³ have shown that the P-450_{C21} genes lie within the HLA region of human chromosome 6.

Although the biochemical basis of the 21-hydroxylase deficiency has never been definitively established, steroid 21-hydroxylation can be catalysed *in vitro* by a combination of two microsomal membrane proteins of the adrenal cortex: P-450_{C21} and a NADPH-dependent cytochrome P-450 reductase⁴. Since only the P-450_{C21} is specific for the reaction, White *et al.*

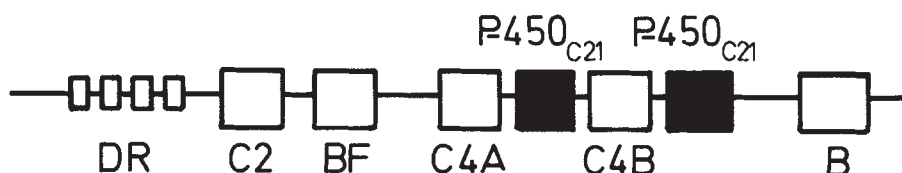
hypothesized that the HLA-linked defect involves a structural gene for this protein. Their test of that hypothesis was facilitated by the knowledge that different forms of the disease are associated with characteristic HLA-B;DR antigens, and that 21-hydroxylase deficiency is in linkage disequilibrium with complement allotypes. For instance, all patients with the haplotype *HLA-(A3);Bw47;DR7* are deficient in both the complement protein C4 and 21-hydroxylase. This led White *et al.* to the additional hypothesis that the genes for C4 and 21-hydroxylase are closely linked and that patients with the Bw47 haplotype may have a large genetic deletion or rearrangement affecting both these genes.

This hypothesis was tested by the use of a cDNA clone coding for bovine P-450_{C21} (ref. 5) to analyse the organization of the P-450_{C21} gene in human DNA. Normal individuals are found to have two P-450_{C21} genes. In the only patient homozygous for both 21-hydroxylase deficiency and HLA-Bw47 antigen, one of the P-450_{C21} genes is deleted. Of patients homozygous for the deficiency but heterozygous for Bw47, one carries a homozygous deletion of a P-450_{C21} gene whereas the others all have heterozygous deletions. The deletions segregate with the Bw47 haplotype over three generations. Thus the *HLA-Bw47* associated 21-hydroxylase deficiency is due to a deletion of a P-450_{C21} gene. However, in the majority of cases where the disease is associated with other HLA-B antigens, the defect is presumably due to much smaller mutations, the nature of which remains to be established.

The elucidation of the molecular genetic basis of congenital adrenal hyperplasia will enable prenatal diagnosis at a much earlier stage than is possible with current methods. This is particularly significant because, in principle, it should be possible to alleviate the condition, at least in female fetuses, by treatment of the mother with dexamethasone.

The prediction of White *et al.* that C4 and P-450_{C21} genes are closely linked is borne out by Carroll *et al.*³, beginning with the demonstration that a segment of human DNA containing the C4 gene hybridizes to a 2.4 kilobase messenger RNA from tissue. Two copies of the gene corresponding to this mRNA are found to lie 1.5 kilobases downstream of the genes for the complement proteins C4A and C4B (see figure). The expression of the gene in the adrenal gland, the size of its mRNA and a partial sequence analysis indicate that it encodes a human P-450_{C21}. (Using a similar approach, White *et al.*⁶ have demonstrated there are two P-450_{C21} genes located immediately downstream of the C4 gene and the related *slp* gene of the mouse, suggesting that a P-450_{C21} gene became linked to the major histocompatibility complex, and was duplicated along with an adjacent C4 gene, sometime before the divergence of mammals).

In contrast to the genes coding for two



Schematic map (not to scale) of the *HLA*-region of human chromosome 6 containing the pair of P-450_{C21} genes. C2, C4A, C4B and BF (factor B) genes encode complement components; DR genes encode Class II HLA antigens and B gene encodes a Class I HLA antigen.

other complement proteins, C2 and factor B, which lie one kilobase apart, 30 kilobases upstream from the C4A genes (see figure) and which probably arose by duplication of a common ancestral gene, P-450_{C21} and C4 are not similar in sequence. The significance of their close association is not clear and there is no obvious explanation for the presence in the major histocompatibility complex of genes coding for a steroidogenic enzyme.

The function of the second P-450_{C21} gene is as yet unknown and it would be of interest to establish whether the two P-450_{C21} genes are highly conserved. It will also be interesting to discover the mechanism responsible for regulating the differential expression of the C4 and P-450_{C21} genes in liver and adrenals.

The 21-hydroxylase involved in congenital adrenal hyperplasia is a member of a 'superfamily' of proteins termed cytochromes P-450. Some members of this family, such as P-450_{C21} itself, are involved in the metabolism of endogenous compounds, whereas others play a central role in the metabolism of foreign hydrophobic compounds, including drugs and carcinogens. Three cytochromes P-450 involved in the metabolism of foreign compounds have been mapped to chromosomes 7 and 9 in mouse^{7,8} and genes coding for

a drug-inducible cytochrome P-450 (ref. 9) have recently been mapped to chromosome 19 in man (E.A.S., I.R.P., M. Davis and S. Povey, unpublished observations). Just as a defect in a P-450_{C21} gene has now been shown to be the cause of congenital adrenal hyperplasia, it is hoped that the availability of cloned DNAs coding for human cytochromes P-450 involved in the metabolism of drugs or chemical carcinogens will lead to a determination of the molecular-genetic basis of inherited variations in the ability of individuals to metabolize these substances. □

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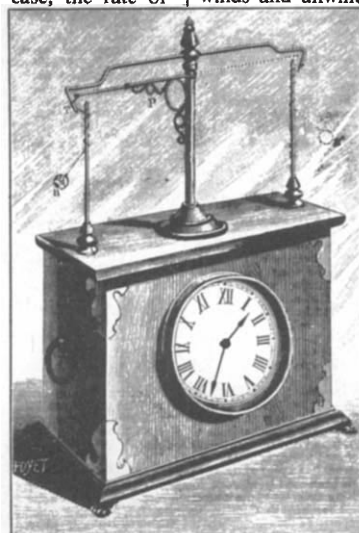
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100 Years Ago A New American Clock

THE accompanying figure from *La Nature* illustrates a new American clock of ingenious construction. It is distinguished from all other clocks by the singular and original form of its pendulum, based on the principle of torsion. A train of wheels gives rotation to a vertical axis, which is seen over the case, the rate of motion of which is to be regulated. This vertical axis supports a sort of bracket, P, to the extremity of which is attached a small bead or ball, B, by means of a thread a few centimetres long. The axis, by the action of the main spring, will turn with a rapid movement, drawing the ball B along with it. To regulate this movement, it is sought to interpose in its path suitable obstacles; this is the object of the horizontal wire terminating in the hooks T, and of the vertical pillars fixed on the case. The bracket P draws the thread in its movement and makes it strike against the arm T; it

is thus arrested, and by virtue of its acquired speed, the ball B winds the thread around the pillar on the left; then follows an unwinding of the thread and a rewinding in an inverse direction, which enable the thread to pass the point T. But in unwinding it strikes a second time against the pillar, winds and unwinds anew, and only succeeds in

passing this double obstacle after four successive windings, twice in one direction, and twice in the opposite direction around the same pillar. The thread thus set at liberty permits the bracket to turn 180° around the vertical axis. After this rotation it encounters two analogous objects placed on the right of the clock, and is delayed a certain time before passing these objects and returning to the pillar on the right. The clock is thus regulated, if not with all the precision of a chronometer, with an approximation said to be sufficient for ordinary use. From *Nature* 31 439, 12 March, 1885.



Mathematics

How bent is a knot?

from Ian Stewart

Anyone who has fiddled about with one of those lengths of plastic-coated spring from which curtains are sometimes hung will have noticed that it is harder to bend the spring into a knotted loop than it is into an unknotted one. Since elastic energy is concentrated on curves, this suggests that a knot is in general more bent than a simple, unknotted loop. The natural context in which to ask whether any mathematical sense can be made of this observation is differential topology, a hybrid creation in which topological notions, such as knottedness and connectedness, are blended with analytical and differential-geometric ideas such as curvature and smoothness. It is one of the deeper areas of today's mathematics, with important successes and applications to its credit. Recent research by N.H. Kuiper and W. Meeks (*Inventiones Mathematicae* 77, 25; 1984) has uncovered some fundamental relations between the topology of a multi-dimensional space (or manifold) and its metric properties, notably curvature.

The notion of curvature goes back to C.F. Gauss in 1828. He defined curvature for surfaces, proved it was an intrinsic property, not dependent on the specific embedding of the surface in 3-space, and applied it to geodesy. It was generalized by B. Riemann to n -dimensional manifolds, and used by A. Einstein as a basic concept in general relativity. The earliest result relating curvature and topology is the Gauss-Bonnet Theorem: the total Gaussian curvature of a surface is $2\pi C$, where C is its Euler characteristic. This is a topological invariant, equal to $2-2g$, where g is the number of 'holes' in the surface. Roughly speaking, the Gauss-Bonnet theorem says that to make holes in a surface you have to put more bend into it. Its importance may be gauged from a remark in M. Spivak's *Differential Geometry* (a classic 5-volume text, described by its author as "The Great American Differential Geometry Book", published by Publish or Perish Inc.). Spivak says: "A whole slew of consequences follows immediately from this spectacular theorem, which expressed a differential geometric quantity in terms of a number which has nothing at all to do with curvature, or even with a Riemannian metric."

Opening remarks about the curvature of a knot can be made precise in a similar way. In 1949, J. Milnor and I. Fary proved that the total curvature of a knot is greater than 4π . For a planar convex loop, the total curvature is exactly 2π . The ordinary trefoil, or overhand knot, can be embedded in 3-space with a total curvature as close to 4π as required, so 4π is the 'best possible' bound.

Instead of knotting a loop in 3-space, topologists are extremely interested in knotting n -dimensional manifolds in N -space. Again, the intuitive expectation is that a knotted surface will have more bend in it than an unknotted one. The amount of bend is once more measured by the total curvature, which is 4π for a sphere and 0 for a torus (in their natural embeddings). Kuiper and Meeks prove a general result which implies, for example, that a knotted torus in 3-space must have total curvature greater than 16π .

To be specific, they relate the total curvature of an n -dimensional manifold M embedded in N -space, to two topological invariants. One of these depends only on the manifold M : it is a relative of the Euler characteristic, and is the sum, β , of the Betti numbers of M . The other depends on the way M is embedded, and measures the extent to which M is knotted: it is σ_1 , the first homotopy excess. (Space limitations preclude a precise explanation of either invariant.) Kuiper and Meeks show that the total curvature must be at least $\frac{1}{2}V(n)(\beta + 4\sigma_1)$, where $V(n)$ is the 'surface area' (or total curvature) of the n -dimensional unit sphere. For example $V(1) = 2\pi$,

$V(2) = 4\pi$, $V(3) = 2\pi^2$, $V(4) = 8\pi^2/3$. There are, however, two exceptional pairs of dimensions. If $n = 1$ (M is a curve) then 4_1 must be replaced by 2_1 . And if $n = 3$, $N = 4$, then v_1 is replaced by the maximum of two similarly defined quantities, calculated for the interior and exterior of M . In these exceptional cases the results are due to R.H. Fox and P. Wintgen.

The proofs rely heavily on new techniques, including Morse Theory (the study of critical points such as maxima, minima, and saddles), the handle decomposition due to S. Smale, the notion of a tight embedding, and Meeks's work on surfaces known as multipants because of their resemblance to generalized trousers for multilegged creatures. The argument is delightfully geometric, as is most of the deeper work in differential topology.

Much of the power of mathematical reasoning derives from its ability to relate concepts that had not been seen to have any connection with each other. The curvature properties of knotted surfaces are an intriguing example of this process and part of a continuing drive to apply metric concepts, such as curvature, to the topology of manifolds. More detailed results along similar lines cannot be far away; meanwhile the view of Gauss and Riemann that curvature is a fundamental property of a space is being vindicated with a vengeance. $\square$

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Molecular genetics

Diverged genetic codes in protozoans and a bacterium

from Thomas D. Fox

THE genetic code, which is the same in *Homo sapiens* as in *Escherichia coli*, is widely thought to have been fixed prior to the divergence of all life forms and so to be universal. Since a mutation changing a codon assignment would effectively cause thousands of mistakes in the proteins translated, it has seemed quite reasonable to assume that such mutations would be lethal. Although studies on mitochondrial genes have shown that the code is not strictly universal, mitochondria could be thought of as exceptions that prove the rule: their genetic systems produce only a very limited number of proteins and so might tolerate changes. Now, some 'real' exceptions have come to light in both eukaryotic and prokaryotic free-living organisms, and the notion of universality will have to be discarded.

In the erstwhile universal code, the codons UAA and UAG mark the termination of translation of a sequence into protein. But in three different ciliated protozoans there is now a strong indication that UAA and UAG encode the amino acid

glutamine. S. Horowitz and M.A. Gorovsky (*Proc. natn. Acad. Sci. U.S.A.* in the press) have determined partial sequences of two genes for histone H3 in the macronucleus of *Tetrahymena thermophila*. (Ciliated protozoans contain a 'somatic' macronucleus and a 'germ-line' micronucleus.) The gene sequences predict the known amino acids of the histone except that each sequence contains two UAA codons at positions that correspond to glutamine in the protein. Thus, UAA must be translated unless both cloned sequences are derived from pseudogenes, which are not translated. This seems unlikely since all 12 mismatches between the two partial H3 sequences produce silent changes. Southern blot experiments show that there are a maximum of four H3 genes in *T. thermophila*, at least two of which must be active since there are two variant forms of the protein in this organism. Thus, while it is possible that the two sequenced H3 genes are silent copies, it is much more likely that UAA actually encodes glutamine.

This conclusion is supported by com-

plementary evidence from another ciliated protozoan, *Stylonychia lemnae*, from which E. Helftenbein (*Nucleic Acids. Res.* 13, 415; 1985) has cloned two different macronuclear genes that code for a protein which is very similar to the α -tubulins of chicken and rat. However, a UAA codon occurs in both genes at a position equivalent to that where the chicken and rat proteins contain glutamine. Since there are only two detectable α -tubulin genes in the *S. lemnae* genome, it seems that the cloned sequences must be active genes. It is hard to escape the conclusion that UAA is translated as glutamine in the α -tubulin gene of *S. lemnae*, which is terminated by a UGA codon, as are all other genes of ciliates examined to date.

Complementary evidence, reported in this issue from two laboratories, comes from the genes encoding variable surface antigens of *Paramecium*. On page 185, F. Caron and E. Meyer report a partial sequence of the single gene of *Paramecium primaurelia* that encodes the G-antigen. With standard codon assignments, the sequence contains no long open reading frame, but by expression in *E. coli* and sequencing of part of the G-antigen sequence fused to *LacZ*, Caron and Meyer were able to determine the reading frame for the G-antigen sequence in *P. primaurelia*. Interestingly, this frame is the only one of the three possible frames to have UAA and UAG codons but no UGA codons, except for a cluster to three near to the 3' end of the transcribed region. Moreover, they show that the erstwhile stop codons are present in the G-antigen messenger RNA by using it as a template for sequencing reactions. Analysis of the A-antigen gene of *Paramecium tetraurelia* has led J.R. Preer *et al.* to similar conclusions. As reported on page 188, no long open reading frames are found in the sequences by standard codon assignments, but one frame predicts amino-acid compositions that are generally consistent with analysis of the protein, and this frame contains UAA and UAG, but not UGA codons. While there are no amino-acid sequence data for these surface antigens that allow definite codon assignments, the amino-acid composition data suggest that both UAA and UAG code for glutamine in *Paramecium*.

All of the ciliate genes examined contain the standard glutamine codons CAA and CAG in addition to UAA and UAG. Thus the latter pair could be thought of as resulting from the fixation of a high-efficiency suppressor gene, leaving UGA as the sole termination codon. How, or when, these events took place is anyone's guess. Either the line leading to ciliates diverged before the standard code was fixed or, perhaps more likely, the changes occurred soon after the divergence of the line from ancestors that used the standard code. This could have happened at a very early stage since ciliates are highly divergent from other eukaryotes: as Horowitz and Gorovsky point out, "Literal use of... molecular

clocks can suggest that ciliates diverged from all other organisms before life originated on earth" (not strictly impossible, but unlikely).

In a prokaryotic organism too, *Mycoplasma capricolum*, divergence of the genetic code has now been demonstrated (Muto, A. *et al. Proc. Japan Acad.* in the press and Yamao, F. *Proc. natn. Acad. Sci. U.S.A.* in the press). This group finds that the sequences of the *M. capricolum* genes coding for the ribosomal proteins S3 and S16, which are highly homologous to S3 and S16 of *E. coli*, contain four UGA codons between them. Three of these occur at positions corresponding to tryptophan in the homologous *E. coli* proteins. Strong support for the idea that these UGAs are translated as tryptophan comes from the sequences, determined by the same authors, of two *M. capricolum* genes for tryptophan-tRNA. One contains a standard 5'-CCA-3' anticodon, but the other has the 5'-UCA-3' anticodon that would be expected for a tryptophan-tRNA able to recognize UGA.

One cannot help but be struck by the fact that the coding of tryptophan by UGA now demonstrated for one species of

Mycoplasma is true also of animal and fungal mitochondrial genes, and that some species of *Mycoplasma* can grow as intracellular parasites. So, are *Mycoplasma* closely related to the presumed prokaryotic progenitor of mitochondria? As exciting as this prospect seems, there are at least two reasons for believing it may not be true. First, in at least some mitochondria of higher plants, UGA does serve as a termination codon (A. Brennicke, personal communication), so the use of UGA to code for tryptophan may not have been a property of the common ancestor of mitochondria, if there was one. Second, unlike some other bacterial species which have cytochrome systems strikingly similar to those of mitochondria, *Mycoplasma* appear to lack cytochromes entirely (Pollack, J.D. *et al. J. Bact.* 146, 907; 1981). It remains to be seen whether non-standard codon assignments will be of value in sorting out phylogenetic relationships or the origins of the genetic systems of intracellular organelles. □

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Laser fusion

Hollow shells, shorter wavelengths

from Roger Evans

SINCE laser-driven inertial confinement fusion (ICF) was declassified in 1972, its somewhat chequered history has centred around the problem of symmetry. Using laser beams, the deuterium-tritium (DT) target pellet has to be compressed to as much as 200 g cm⁻³, an enormous compression that distinguishes the ICF pellet from the hydrogen bomb. In both cases, the DT fuel must be heated to the thermonuclear ignition temperature of 10⁸ K, but the much larger mass of fuel in a bomb need not be compressed to the same degree in order to achieve the combination of density, temperature and duration required to permit fusion — the equivalent of the Lawson criterion. Two recently published papers have shown major advances in solving the symmetry problem and give a renewed vigour to inertial confinement research^{1,2}.

In the original paper on laser-driven ICF, Nuckolls³ suggested imploding a small solid pellet of DT fuel, but later Kidder⁴ showed that the laser power required could be significantly reduced if the fuel were a hollow spherical shell instead of a solid. For a given fuel mass, this would be larger, its implosion time would be longer, the laser energy could be delivered over a longer time and the power level would be reduced. The extent of this gain depends on the ratio of the shell thickness to its radius, known as the 'aspect ratio'; large thin shells require less laser power to achieve the same degree

of compression.

This advantage is not without its drawbacks as the imploding hollow shell suffers from the Rayleigh-Taylor fluid instability. This occurs whenever a dense fluid is supported by a light fluid under gravity. Any distortion of the fluid interface is amplified, resulting in 'spikes' of dense fluid falling while 'bubbles' of the light fluid rise upwards. In laser-imploded targets the gravity is provided by the acceleration, the tenuous plasma heated by the incoming laser light is the light fluid, and the solid shell of imploding material is the heavy fluid. The overall growth of the Rayleigh-Taylor instability scales as the square root of the shell aspect ratio, so that impossible levels of smoothness and illumination uniformity are required for shells of high aspect ratio.

The advantages gained from reducing the laser power with large aspect-ratio shells are not simply the economies of reducing laser costs but are quite fundamental, because light of lower intensity has a more favourable interaction with the target plasma. As the laser intensity increases, a greater proportion of energy goes into removing material from the pellet at the expense of compression efficiency. A major recent advance was the realization that shorter laser wavelengths allow the use of higher light intensities while maintaining good coupling efficiency, because the shorter wavelength light penetrates to

higher densities and thus ablates more material at slower velocity, resulting in more efficient compression. However, coupling the laser energy into high density material reacts back on to the problem of symmetry. No real laser system is ever likely to produce the one per cent symmetry (defined as the fluctuation in laser luminosity over the target's surface) needed to drive a high-density implosion, and some reliance is placed on 'smoothing' the non-uniform illumination within the target atmosphere. Since the short wavelength laser light penetrates further, there is clearly less atmosphere to effect the smoothing and the requirement for illumination symmetry is correspondingly tighter.

The growth of the laser-driven Rayleigh-Taylor instability in flat foil targets has now been measured by workers at the US Naval Research Laboratory¹. Although this is not the first such measurement², it is the first time that two-dimensional images have been obtained. It was made with a flat-foil target, the rear surface of which was grooved like a diffraction grating to act as a 'seed' for the Rayleigh-Taylor instability, accelerated with about 500 J of laser energy at 1.06 μm wavelength. When the instability grows, the line of sight passing through the 'spike' contains much more material than an adjacent sight line passing through the 'bubble' and gives rise to differences in transmission in a flash X-ray radiograph. Comparing the results with sophisticated computer models shows that the growth rate is about half of the classical value. It has been suggested that the reduction is due to the ablation of fluid across the Rayleigh-Taylor unstable region, which gives rise to the classical phenomenon of vortex shedding. If correct, this is good news for short-wavelength laser users because the increased ablation flow should give rise to a further reduction in the Rayleigh-Taylor growth rate.

A second paper², from the Osaka group in Japan, reflects the very heavy involvement of the Japanese in fusion research, and particularly in laser fusion, where their Gekko XII glass laser is currently the most powerful in operation anywhere⁶. This group has tackled the problem of uniform illumination in a very imaginative way: instead of trying to achieve the most uniform illumination, use has been made of the paradoxical finding that really bad laser beams can be more effective than good ones, because the target is most sensitive to a particular range of spatial periodicities. Very short wavelength non-uniformities can be removed by the smoothing of the target atmosphere, while wavelengths that are very long compared with the target radius grow quite slowly and are not very damaging.

The Japanese solution is to divide the laser wavefront into a multitude of small rectangular beamlets and to introduce phase delays between adjacent beamlets with a 'random phase mask'. Each beamlet is sufficiently small that, when focused on

to the target, its diffraction-limited spot is about equal to the target size; the target sees a large number of diffraction-limited, and therefore smooth, beams. Since the beams are all coherent with each other, they interfere in the target plane and give rise to a scrambled intensity pattern with a very short characteristic wavelength. The Japanese group has performed several target experiments using the random phase mask and has reported favourably on its effectiveness.

The combination of these two new results and other successful implosion ex-

periments reported at the recent International Atomic Energy Agency meeting in London⁶ renews enthusiasm for short-wavelength lasers as fusion drivers. Further progress is eagerly anticipated. □

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Prehistoric astronomy

Evidence from a new site

from Clive Ruggles

THE ashes of an old and fiery debate on the reality of 'megalithic astronomy' will be stirred by the paper on page 158 of this issue, in which MacKie *et al.* describe, and provocatively interpret, a newly-excavated site in Scotland.

Five years ago it was possible to believe that 'megalithic astronomy' was becoming a sterile field of research. Interest had waned in Stonehenge — for many years the epitome of a supposedly astronomical site — because many highly speculative interpretations, some of which had received wide publicity, had largely been refuted. The long debates generated by one or two high-precision astronomical alignments at a few 'classic' sites, such as Ballochroy in Kintyre and Kilmartin (Temple Wood) in Argyll, were also slackening off, essentially for lack of evidence to assess whether the alignments were deliberate or simply arose by chance.

The meticulous statistical analyses of Alexander Thom, combining evidence from many sites, were then claiming a level of precision for prehistoric lunar observations as little as two minutes of arc, which is quite beyond what seems to be possible, even after observation periods of 200 years. The objectivity of Thom's selection of data was being called into question; and, as a result, so were a number of his claims from earlier analyses, such as the existence of a calendar formed by dividing the solar year into sixteen equal parts, and of indicated horizon foresights marking 'calendrical' declinations.

These claims were starting to be the subject of thorough reassessment by critics from the numerate disciplines, although for many archaeologists this reassessment was redundant. For years they had regarded with grave suspicion the conclusions of astronomers, engineers and the like who, fascinated by the idea of prehistoric astronomy, specifically sought out evidence for it. The dangers in so doing are twofold. First, we may miss or misinterpret a wide range of other evidence bearing upon the purpose of a site and its builders.

Second, we may unwittingly project our own ideas of 'achievement' on to the culture whose material remains we are studying. Such ethnocentrism seemed blatant in the approach of those who talked of 'megalithic observatories' — sites considered to have been primarily constructed for careful observation of the Sun, Moon and stars — and led many to dismiss out of hand the conclusions of Thom and others.

Recent developments have changed general attitudes towards prehistoric astronomy. First, there has been a movement away from the consideration of individual sites, towards a more archaeologically-informed assessment of evidence from coherent combinations of sites. Statistical analyses now pay greater attention to the objective selection of data, and the whole approach recognizes that astronomical considerations will be only one amongst a number of factors influencing the placement and design of a site. This sort of approach has uncovered evidence of low-precision lunar alignments amongst linear settings in western Scotland and the Recumbent Stone Circles of Aberdeenshire. At the same time, severe doubt has been cast upon the hypothesis of observations more precise than one or two degrees (including Thom's solar calendar).

Second, archaeologists have begun to consider astronomical practice as but one aspect of ritual activity undertaken at ceremonial sites. Thus by relating evidence of lunar orientation to excavated evidence at the Recumbent Stone Circles, Aubrey Burl has concluded that the sites had a ceremonial significance in which lunar symbolism was important. The increased communication between those trained in the sciences and those trained in the humanities has lain the seeds of more general attempts to study and understand prehistoric astronomy, not in isolation but more properly in relation to its social context.

While statistical analyses must continue to play a crucial role, investigations of particular sites still have something to

offer, particularly if their excavation is designed to test a particular astronomical hypothesis rather than simply to collect data. A long-standing proponent of this approach has been Euan MacKie, who in 1970 and 1971 excavated a structure at Kintraw, Argyll, claimed by Thom to be an observing platform. Although widely quoted, the evidence from the excavation was unfortunately inconclusive, as MacKie himself now admits. In this issue of *Nature* he and his co-authors discuss evidence from Minard — another site in Argyll — where archaeological excavation was seen as testing an astronomical hypothesis. Perhaps the most striking result is that, unlike Kintraw, Minard indisputably has platforms that have been levelled by man; moreover, the main features at the site have been fairly securely radiocarbon dated to the mid-second millennium BC. Together, the various archaeological discoveries indicate that Minard was not a domestic, defensive, industrial or funerary site, but one intended, perhaps almost exclusively, for ritual use. Furthermore, both the striking distant horizon to the north-east, roughly in the direction of midsummer sunrise, and the general orientation of the site in this direction, seems to indicate that solar orientation was a deliberate and crucial function of the site.

Those interested in prehistoric astronomy will still have many questions to raise about the precise interpretation of Minard. In particular, many will dispute whether, on statistical grounds, the claim that it was used for high-precision observations (supporting Thom's 'calendrical' hypothesis) is really borne out. The north-eastern horizon foresight does not fall



Minard, Argyll. Was the site used for astronomy in megalithic times?

exactly at the solstice, and any feature occurring within a few degrees of the solstitial position is susceptible to the 'halving the difference' argument used by MacKie *et al.* (*viz.*). There are similar problems when defining the 'calendrical' equinox. Thus the evidence for high-precision astronomy is perhaps not as clear as MacKie's paper might lead us to believe; nonetheless Minard is surely destined to become an important site, evidence from which will generate considerable debate and, I hope, a few fresh hypotheses. □

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Genetic analysis

Of man and mouse

from P.N. Goodfellow

ON page 181 of this issue B. Robert *et al.*¹ describe their determination of the chromosomal localization of the genes that code for several muscle proteins of the mouse. These results will engage those studying co-ordinate regulation of muscle proteins and those interested in the evolution of gene families. More importantly, by exploiting the species differences between *Mus domesticus* and *Mus spretus*, Robert *et al.* introduce a new technique of mapping genes to chromosomes that may well become a standard tool for genetic analysis of the mouse.

The laboratory mouse has many advantages over man for genetic analysis, including a short generation time, plentiful genetic markers, and no restrictions on brother-sister or parent-child matings. So great were the advantages of working with mice that many geneticists believed that the proper study of man was mouse, a view which was encouraged by the

development of inbred², congenic³ and recombinant-inbred strains⁴. Without these tools, it is doubtful whether many familiar systems, such as the genetics of transplantation and immune response, would yet have been unravelled.

Two advances have made the direct study of human genetics as profitable as the study of the mouse. First, the introduction of somatic cell techniques freed human genetics from the disadvantage of the long human life span and exploited the species differences between humans and rodents to provide genetic markers; using this method over 400 loci have been assigned, directly or indirectly, to human autosomes⁵, whereas only two or three had been assigned before the application of somatic cell genetics⁶. Second, in recent years, molecular biology has provided an almost limitless supply of genetic markers in the form of restriction fragment length polymorphisms (RFLP) defined by cloned

DNA probes (see ref.7 for a recent review and ref.8 for the latest advance). These markers have rejuvenated classical genetic approaches and have already contributed to the chromosomal localization of loci associated with muscular dystrophy⁹ and Huntington's disease¹⁰.

Mouse genetics has also benefitted from somatic cell techniques and molecular biology but because of the related origin of the common inbred mouse strains^{11,12} and the small number of strains available², it more difficult in mouse than in man to find RFLPs that can be used as genetic markers. To overcome this deficiency, Robert *et al.* mated an inbred strain of the laboratory mouse, *Mus domesticus*, to an inbred strain of the related mouse, *Mus spretus*¹³. The two progenitors of the laboratory mouse, *Mus musculus* and *Mus domesticus*, have been separated from *Mus spretus* for about three million years¹⁴, which is sufficient time for there to have been enough divergence in DNA sequences to generate potential RFLPs in any cloned mouse gene.

In the wild, *Mus domesticus* and *Mus spretus* probably never mate with each other¹⁵, but, under carefully provided laboratory conditions, fertile female offspring can be produced¹³ and back-crossed to the parental strains¹⁶. (The male offspring of the between-species cross are infertile, as predicted by Haldane's law¹⁷). As demonstrated by Robert *et al.* for myosin alkali-light chain genes¹, linkage between the RFLPs and other genetic markers can easily be followed in the back cross and used for gene mapping.

For some genetic studies, including deliberate mutagenesis, the mouse will always be the mammal of choice. Ethylnitrosourea can induce mutations at the incredibly high frequency of one mutant at each locus for every thousand offspring¹⁸. This means that saturation mutation mapping¹⁹ and the deliberate creation of mouse models for human disease are both feasible. Perhaps the proper study of man is both man and mouse. □

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Histidine-rich protein of *Plasmodium lophurae*

SIR — Ravetch *et al.* have reported some very interesting findings on the primary structure and genomic organization of the histidine-rich protein of *Plasmodium lophurae*¹. Their observation of the presence of multiple copies of the signal-peptide sequence in the genome of *P. lophurae*, along with the increasing evidence for the presence of histidine-rich sequences in additional malarial polypeptides, pose numerous challenging questions. The fragmentary available information seems to suggest that some related sequences have been conserved through the evolution of apparently functionally different malarial polypeptides.

The recent literature on histidine-rich proteins of malaria parasites has certainly had inconsistencies and can seem confusing to newcomers to the field. This creates all the more reason for factual reporting. Until 1983, the unique and fully characterized histidine-rich protein of *P. lophurae* was referred to as the 'HRP' of *P. lophurae* in all the malarial literature. Although in my publications a clear distinction has been set between HRP and other related polypeptides, there have been several references to undefined histidine-containing malarial polypeptides as 'HRP'.

Perhaps by confusing fact with hypothesis or different histidine-rich polypeptides, Ravetch *et al.* have taken their interesting findings totally out of the context of *P. lophurae*. They cite my paper² as their source for the statement that the histidine-rich protein "is located in a membrane-bounded compartment that forms part of the specialized parasite organelles implicated in erythrocyte invasion". Technically, these specialized organelles are the rhoptries of merozoites, the infective forms. However, the facts presented in the paper cited show that the protein was purified from cytoplasmic granules of trophozoite stages of *P. lophurae*². As the parasites develop, the granules increase in number and eventually become segregated inside the residual food vacuole. When the host cells lyse and merozoites are liberated, the granules remain enclosed in the food vacuole which is also released.

These facts offer no grounds for speculating that "if histidine-rich protein becomes a surface molecule of *P. lophurae*, it must be secreted" or "if secretion does occur, it would be followed by disassembly of protein aggregates before association with the parasite surface". As shown in the electron micrograph of my paper, the protein aggregates in the form of electron dense granules while it is within the rough endoplasmic reticulum. Therefore, the logical interpretation of the observed structural features which are characteristic of secretory proteins has to be sought in the context of the synthesis and translocation of the polypeptide within cytoplasmic compartments of the parasite, rather than

secretion to the surface. I have described a secretory polypeptide that is related to the histidine-rich protein of *P. lophurae* but it is in another species of malaria. Also, the idea of the possible relationship of a histidine-rich protein to the infection process does originate from my paper entitled 'Does a histidine-rich protein from *Plasmodium lophurae* have a function in merozoite penetration?'³. This was published as a hypothesis and has been neither proven nor disproven. I would be delighted to see it proven.

Ravetch *et al.* seem confident of the accuracy of the tandemly repeated sequence. In comparison with the known amino acid composition of the mature polypeptide, the deduced sequence shows significant differences in proline and aspartic acid content. In our hands, the histidine-rich sequences of both genomic and cDNA clones were totally unstable in *Escherichia coli*.

Finally, the statement that "other investigators have been unable to reproduce" my results on the protective effect of the histidine-rich protein cannot be supported by the literature cited. The use of altered experimental protocols does not imply "reproduction".

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Climatic effects of volcanic eruptions

SIR — Major volcanic eruptions in the future will cause, as they have in the past, significant short-term climatic changes, and these changes may have severe social consequences¹⁻⁵. Recent discussions of the climatic effects of volcanic eruptions have emphasized their importance as analogues of the consequences of nuclear war^{1,2}. While studies of volcanic eruptions can make an important contribution to assessing the effects of a possible nuclear war, we fear that to use volcanic studies in this way draws attention away from the very serious problems that the eruptions themselves present. A nuclear war remains, fortunately, only a hypothetical possibility. But it is certain that climate-altering volcanic eruptions will take place in the future, although the frequency of these is not well established⁶.

The most recent such eruption, that of Tambora 1815, caused widespread disruption to agriculture in North America and Europe, leading to economic crises, famine and outbreaks of disease, whose effects have been summarized by Stommel and Stommel⁷. The stratospheric aerosol loading produced by Tambora was equivalent to a peak optical depth of about 1 (ref. 3). Much the largest effects of

historic times appear to have been associated with a volcanic aerosol of unknown provenance of AD 536, calculated to have had an optical depth of about 2.5, which had severe effects on agriculture in the Middle East⁴. Several other historic eruptions have had less severe but still significant effects, notably the Laki eruption of 1783⁸.

Since 1815, the world's population has increased many-fold. The continuing famines in the arid and semi-arid regions of northern Africa demonstrate the disastrous human problems that result when relatively minor climatic changes affect marginal areas which are already at or near their maximum sustainable populations. An eruption of the magnitude of Tambora taking place at the present day could have catastrophic effects on agriculture, causing crop failures and shortages world wide, with substantial social and political implications. The fact that the frequency of such large volcanic eruptions may be rather low³ offers little comfort: a Tambora or even a Toba magnitude eruption could take place tomorrow. Furthermore, the climatic effects of volcanic eruptions are a function of their ability to produce stratospheric aerosols as well as their absolute magnitudes^{9,10}. Eruptions smaller than that at Tambora could therefore cause acute problems, especially given the present human pressure on food supplies.

Whilst not wishing to minimize the importance of research on the climatic effects of nuclear war, we suggest that some effort should be diverted from the hypothetical to the inevitable, and that attempts be made to determine the possible climatic and agricultural effects of future large and intermediate magnitude volcanic eruptions. Governments and international agencies might then be in a position to prepare contingency plans capable of dealing with the likely eventualities.

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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a rather technical character which are not provoked by articles or letters previously published (where Matters Arising remains appropriate).

Origin of strong interplanetary shocks

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Observations of 900 radio sources have been used to study the shape and motion of large-scale interplanetary transients associated with shock disturbances at 1 AU. The variations of plasma density and speed and the zones in the solar atmosphere from which the transients originate suggest an origin related to intermittent flows of enhanced speed from coronal holes at mid-latitudes on the Sun.

A BETTER understanding of the mechanisms involved in the genesis of strong interplanetary shocks is important not only to solar physics, but also for practical reasons concerned with disturbances to communication systems, electrical power transmission, geomagnetic surveys and with possible radiation hazards to astronauts^{1,2}. New information about the shape and dynamics of large-scale interplanetary transients is now becoming available from radio astronomical measurements on a large grid of 900–2,500 radio sources whose scintillation can be used to monitor perturbations of plasma density along many lines of sight through the interplanetary medium³. The ability to map the disturbances and to estimate their speed and direction of travel is helpful in locating their sources at the Sun. Here, we present observations of interplanetary transients associated with three of the strongest shocks that reached the Earth during 1978–79: about 70 other examples were found during this period of high solar activity and will be presented in detail elsewhere.

Plasma density

Our observational methods and techniques of data reduction have been described before^{3,4}. For each source we measure ΔS , the r.m.s. variation of flux density at 81.5 MHz integrated over the band 0.1–3.0 Hz during an interval of about 30–50 s at meridian transit. ΔS varies systematically with solar elongation in a manner which can be determined accurately from daily observations continued for about 1 yr. To specify day-to-day perturbations about the mean, we define an enhancement factor $g = \Delta S / \overline{\Delta S}$, where $\overline{\Delta S}$ is the average value for a given source at a particular elongation.

It has long been known that variations of g are correlated strongly with variations of total plasma density along the line of sight to a source³. It has also been suggested that enhanced turbulence, rather than enhanced density, may sometimes be correlated with increased scintillation⁵. This possibility is important for the present study, where lines of sight pass frequently through post-shock flows, so we have performed an analysis to check the correlation under a variety of interplanetary conditions. The large grid of 900–2,500 sources under daily observation enables this to be performed more accurately than had previously been possible.

The region of the solar plasma responsible for scintillation at 81.5 MHz is sketched in Fig. 1a. On 30 occasions during 1978–81, when we were fairly certain that stable conditions persisted along most of the relevant portions of appropriately located lines of sight, we obtained the results shown in Fig. 1b. The observations refer to lines of sight within 30° of the ecliptic at elongations near 90°, so that the best comparison could be made with near-Earth spacecraft data; a full account of this work will be given elsewhere. Occasions were selected when the *in situ* density remained approximately constant for at least 24 h and the average value of g was obtained typically from observations of 50–100 sources. The conditions giving rise to perturbed plasma density included both relatively stable co-rotating streams and more sudden transients often associated with shocks.

Our results are shown in Fig. 1b, which demonstrates the strong correlation between plasma density (N) and g . Over the

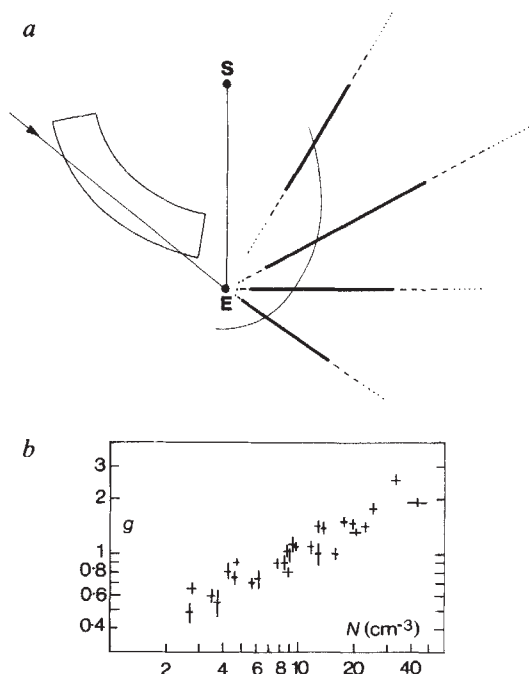


Fig. 1 *a*, Schematic diagram of scintillation weighting functions along various lines of sight normalized to unity at the locus of maximum scattering (curved line). The ranges are: 0.5–1.0 (heavy line), 0.25–0.5 (dashed line), 0.1–0.25 (dotted). S, Sun; E, Earth. *b*, Correlation of scintillation enhancement factor (g) with *in situ* plasma density N . Each point is the average of observations of 50–100 sources at elongations near 90°.

range $3 < N < 40 \text{ cm}^{-3}$ the observations satisfy a relation $g = (N \text{ cm}^{-3}/9)^{0.52 \pm 0.05}$. During the 2-yr period near sunspot maximum, we found no evidence for enhanced or decreased scintillation not associated with corresponding variations of density. We therefore conclude that scintillation can be used with confidence to monitor plasma density along the line of sight to a source under widely differing heliospheric conditions.

The technique is best suited to the detection of large disturbances which cover the heavily weighted portions of the lines of sight. Shell-type transients thinner than $\sim 0.05 \text{ AU}$ might be undetectable. As our observations are made at meridian transit, there is also a finite chance that some disturbances could pass through the zone of detectability unobserved if they travelled faster than $\sim 1,000 \text{ km s}^{-1}$.

Observations

The transients to be discussed caused sudden-commencement magnetic storms when they reached the Earth, followed by the strongest geomagnetic disturbances ($A_p > 100$) during August 1978–September 1979 and were also accompanied by energetic particles.

Contour maps showing the variations of g across the sky as the transients propagated outwards from the Sun are shown in

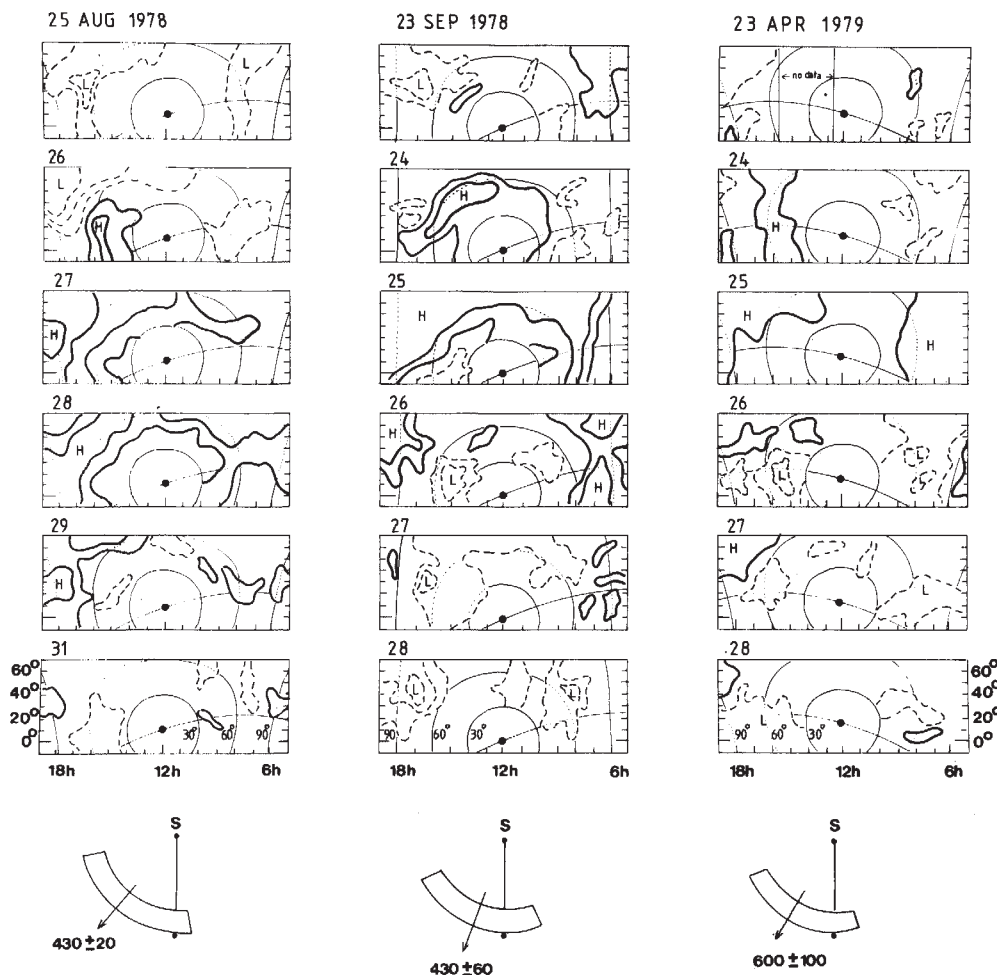


Fig. 2 Sky maps of scintillation enhancement (g) for three transients; coordinates: x , hour angle; y , declination. The contour levels for $g > 1$ are 1.25, 1.5, 1.75 (heavy lines) and for $g < 1$ are 1/1.25, 1/1.5, 1/1.75 (broken lines). Areas of highest (H) and lowest (L) values are indicated. Approximate shapes and speeds of enhanced density transients derived from the maps are also shown.

Fig. 2. Most of the information about transients is obtained from observations within 1 AU and so the maps have been plotted only to ± 7 h from local noon. For qualitative interpretation, note that lines of sight near elongation 90° monitor disturbances as they pass the Earth. Mapping is not continued within 30° from the Sun as this is the zone of strong scattering where g is expected to show an inverse correlation with density. Significant perturbations of g are not usually observed in this region.

We have shown previously how a comparison of observed maps with theoretical maps computed for simple models of density perturbations can be used to deduce the density structure in three dimensions^{4,6}. The period 1978–1979 had unusually high solar activity and most of the disturbances that we observed approximated to outward-moving spherical shells or co-rotating density structures associated with relatively long-lived high-speed solar wind streams. The approximate shapes of the enhanced density zones of the three transients are shown in Fig. 2. It is possible that the radii of curvature of the disturbances are less than indicated (although they must exceed 0.5 AU), but there is no doubt that they cover a wide range of helio-longitude and latitude. The typical solid angle is of the order $\pi/2$ sr; the precise latitude range is less easily determined and will be discussed below. The radio source grid is sufficiently fine that the outer boundaries of the transients can usually be located to an accuracy of $\pm 5^\circ$ and the day-to-day positions can then be used to estimate their speeds and arrival times at the Earth. Some other features on the maps, such as the low-density zones on 25–26 August, the structures at elongations $> 45^\circ$ on 23 September and the high-density zone at elongation $> 90^\circ$ on 24 April are associated with other transients or longer-lived co-rotating streams and will be described elsewhere.

Figure 3a shows the expected position as a function of time of the outer boundary of enhanced scintillation computed for

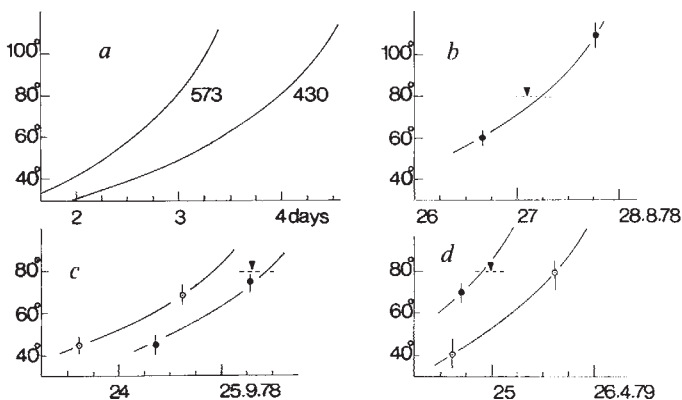
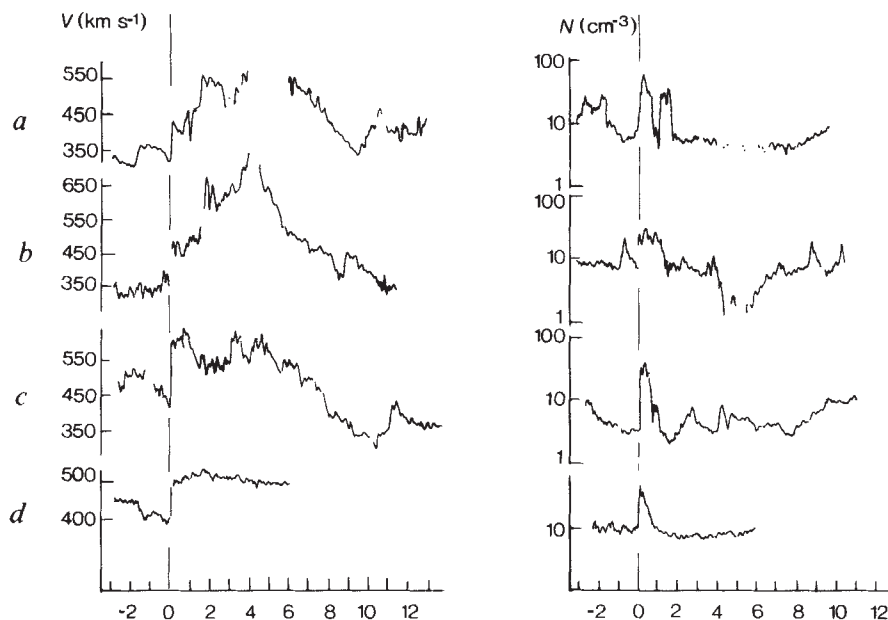


Fig. 3 *a*, Solar elongation of the outer boundary of enhanced scintillation computed for a spherical shell of enhanced density travelling at 30° to the Sun/Earth line. The starting time at the Sun is 0 days. *b–d*, Observed positions on the boundaries of transients. *b*, Outer boundary near the ecliptic to the east (●); *c*, outer boundary above the ecliptic (○) and on the ecliptic to the west (●); *d*, outer (●) and inner (○) boundaries near the ecliptic to the east. The times of sudden-commencement magnetic storms are indicated (▼). The curves fitted to the observations correspond to the speeds shown in Fig. 2.

a spherical shell of increased density of thickness 0.2 AU, covering a range of $\pm 36^\circ$ in helio-longitude and latitude and centred on a direction at 30° to the Sun/Earth line in the ecliptic. Constant speeds of 430 km s^{-1} and 573 km s^{-1} were assumed, corresponding to transit times of 4 and 3 days from the Sun to the Earth. The weighting of scintillation along the line of sight is such that the curves for elongations $< 100^\circ$ are not sensitive

Fig. 4 The solar wind speed and plasma density observed by spacecraft near the Earth. The temporal variations are aligned with respect to the shocks (vertical line) at zero time. The arrival times are: *a*, 0300 UT, 27.8.78; *b*, 0718 UT, 25.9.78; *c*, 2357 UT, 24.4.79; *d*, a superposed epoch plot of 103 shocks observed during 1971–78.



to the direction of travel, provided that the transient is centred on a line within $\sim 45^\circ$ of the Sun/Earth line. The models indicate that the outer boundary is seen at an elongation near 80° when the transient is about to hit the Earth. Some observed positions of the boundaries of the transients mapped in Fig. 2 are plotted in Fig. 3*b–d*. The measurements refer to positions near the ecliptic, either to the east or west of the Earth. The September transient was clearly directed above the ecliptic and in this case we have also shown the location of the outermost boundary at high helio-latitude. Radio interference prevented observations of the front of the transient on 23 April and here we have made use of positions of the inner boundary. The curves fitted to the points are model curves (as in Fig. 3*a*) with suitable velocity scaling.

The times at which the transients reached 1 AU in the ecliptic are in good agreement with the onset of sudden commencement magnetic storms, indicating that the shock fronts must be close to the outer boundaries of the high-density shells causing enhanced scintillation. To complete the observations we display in Fig. 4*a–c*, the solar wind speed and plasma density observed by spacecraft close to the Earth⁷ during the same periods.

Solar origin of the transients

To seek possible sources of the transients, we projected them back to the solar surface assuming transit times of 3 days for August and September and 2 days for the faster April transient. These delays are less than the 4- and 3-day transit times appropriate to our derived speeds and were chosen to allow for slight deceleration in accordance with spacecraft observations of the April event⁸ for which the mean speed between 0.41 AU and 0.99 AU was 789 km s^{-1} , whereas the speed at 1 AU was 608 km s^{-1} .

The zones so defined are shown as circles on the synoptic maps in Fig. 5*a–c*. The greatest uncertainty concerns the helio-latitudes, as in two cases the maps do not extend sufficiently far below the ecliptic. It is probable that the August transient was directed slightly above the ecliptic, whereas that of April could have been below. Because this could not be confirmed they have been centred at 0° . Also shown on the synoptic maps are the outlines of coronal holes obtained from He 10,830 Å images (Solar Geophysical Data), the positions of notable solar flares or disappearing filaments discussed in connection with the same transients by other workers and the projected ranges in helio-longitude at the Sun of the high-speed streams ($V > 450 \text{ km s}^{-1}$) observed by near-Earth spacecraft⁷. The latter estimates were based on an assumed transit time of 3 days.

Figures 4 and 5 show that the shells of high density are followed by high-speed streams which originate from roughly the same zones on the Sun and subsequently persist for at least 6 days. During the September event the stream showed a further acceleration some 4 days after the initial shock. The same streams are also seen in Fig. 2, where they correspond to zones where $g < 1$, in accordance with the usual reduction of plasma density in regions of higher than average speed. Note that averaging along the line of sight in our data smooths out some of the complexity seen in spacecraft measurements at a single point and also that the density was already low before the April transient. One feature common to all the transients is that they erupted from zones containing coronal holes at moderately low helio-latitude. As it has been established that long-lived coronal holes, such as those that existed during 1974–75, are sources of relatively stable high-speed streams, we believe that our observations are most easily explained by supposing that less stable holes, typical of the ascending phase of the solar cycle⁹, are the sources of variable speed flows. If the wind from such a hole increased from, say, 450 km s^{-1} to 600 km s^{-1} on any timescale shorter than $\sim 12 \text{ h}$, the faster flow would catch up the slower flow ahead of it, thus generating a compression zone of enhanced density and a steepening velocity gradient which could develop into a shock at 1 AU. Theoretical modelling with reasonable velocities has demonstrated the general characteristics of the expected perturbations¹⁰.

The same coronal hole was associated with both the August and September transients. It first appeared on Carrington Rotation 1671 and persisted for three rotations. Clouds prevented observations on 23–24 August when the transient high-speed flow should have commenced but a significant increase of area ($\sim 25\%$) occurred during 22–25 August (J. W. Harvey, personal communication). Such changes are fairly common and might signal increases of solar wind speed. There are two possibilities for the source of the April transient which has been associated previously⁸ with the disappearing filament shown in Fig. 5*c*: (1) the hole at longitude 30° and latitude 25°N ; (2) the complex hole structure in the Southern Hemisphere. On the following rotation (CR 1681) a large hole at similar longitude extended upwards from the southern pole to latitude 15°S following the tendency for mid-latitude holes to merge with holes at higher latitudes as they overtake them under differential rotation⁹. Coronal hole activity in the south is the most probable source of the transient.

Precise alignment of the projected zones of origin with coronal holes is not expected. High-speed streams at 1 AU typically extend over wider ranges of longitude than those of the hole

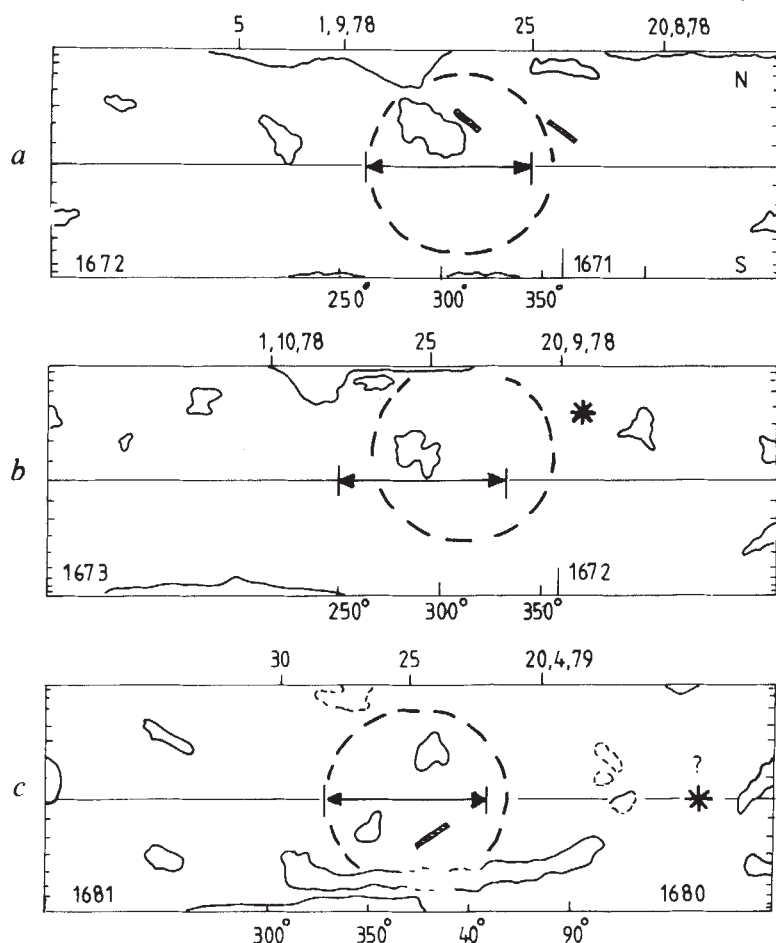


Fig. 5 Predicted areas (dashed circles) from which three transients erupted plotted on synoptic maps showing the boundaries of coronal holes obtained from He 10,830-Å images. Arrowed zones on the Equator denote the predicted longitude range of high-speed streams of speed $\geq 450 \text{ km s}^{-1}$. Date marks correspond to CMP at 0000 UT. The locations of flares (stars) and disappearing filaments are indicated.

boundaries seen in the He 10,830-Å images and little is known about the divergence of streams near the Sun. Again, the co-rotation of transient high-speed streams after their eruption will generate enhanced density at the western edges of the streams¹¹; this could account for the small (and possibly insignificant) systematic westerly displacement of the high-density zones with respect to the streams seen in Fig. 5.

Sudden disappearances of solar filaments have been associated with the transients of August and April and their positions are shown in Fig. 5 (refs 8, 12 and J. W. Harvey, personal communication). Both the timing and location of these events are consistent with our observations; it is reasonable to speculate that large-scale disturbances of the solar wind will be accompanied by major changes in the coronal magnetic field that could destabilize the filaments.

Solar flares have also been associated with two of the transients¹³ although that for the April event was later discounted¹⁴. Our observations disagree with the suggested flare-source for the September transient, shown in Fig. 5b, where there is a large discrepancy in position and the timing is also inconsistent. The flare occurred at 0940 UT on 23 September while the outer boundary of the transient was observed at a distance of 0.7 AU at 1500 UT on the same day. If the flare triggered the disturbance, the average speed must have been $\sim 7,000 \text{ km s}^{-1}$, which is unreasonably high.

We discussed previously another flare discrepancy³ and our observations during 1978–81 contain further examples, raising

the question of whether flares are as effective in causing shock-disturbances at 1 AU as is sometimes assumed¹³. Figure 4d shows a superposed epoch plot of 103 shock events at 1 AU, the sample constituting all the shocks observed during 1971–78 on the IMP 6, 7, 8 spacecraft¹⁵. The average properties are very similar, though less extreme, than the major transients we have described. In particular, the high-speed flow behind the shocks lasts, on average, for 5–6 days. Both the duration of the flows and the slow increase of velocity to a peak 1–2 days after shock passage are more similar to the behaviour of transient solar wind streams than to the expected properties of shocks induced by the sudden release of large amounts of energy in the lower corona¹⁶. In the latter case, models predict a swifter return to ambient conditions.

The new cause that we suggest for major disturbances does not eliminate the possibility of correlations between flares and shocks at 1 AU. It is probable that the emergence of more energetic solar wind flows is accompanied by changes in the coronal magnetic field structure which could trigger a variety of sudden events in the solar atmosphere including flares and eruptive prominences. But these would be peripheral to the main disturbance. The important aspect of our theory concerns the primary source of energy for large-scale interplanetary transients. This we identify with the mechanism that accelerates high-speed streams and not with the impulsive release of magnetic free energy in the chromosphere and the corona.

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Fractal growth of viscous fingers: quantitative characterization of a fluid instability phenomenon

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What happens when one attempts to push water through a fluid of higher viscosity? Under appropriate experimental conditions, the water breaks through in the form of highly branched patterns called viscous fingers. Water was used to push a more viscous but miscible, non-newtonian fluid in a Hele-Shaw cell. The resulting viscous finger instability was found to be a fractal growth phenomenon. Reproducible values of the fractal dimension d_f were found and were interpreted using a modification of the diffusion limited aggregation model.

WHEN one attempts to use a low viscosity fluid (like water) to push high viscosity fluid (like oil), there arises a fluid instability phenomenon termed the 'viscous fingering instability' in which the low viscosity fluid forms characteristic fingers extending well into the high viscosity fluid¹⁻⁴. In addition to its obvious interest in enhanced oil recovery by water flooding, and other practical applications, this phenomenon is of considerable basic interest and is not understood at present. It may be studied in detail in the laboratory using a Hele-Shaw cell, which is a pair of transparent plates separated by typically 0.5 mm. The space between the plates is filled with high viscosity fluid and the low viscosity fluid is injected. Normally, the interfacial tension between the two fluids is high and the low viscosity fluid forms non-fractal patterns that resemble the fingers of a glove^{1,2}. Fractal phenomena are found to be associated with a range of random systems⁵⁻⁸. For this reason it is of interest to inquire whether or not there exist experimental conditions under which viscous fingers obey a fractal symmetry.

Experiments

Fractal structures in nature typically occur when randomizing effects dominate over stabilizing effects. Hence to find a fractal structure we must eliminate stabilizing effects so that the randomness can dominate a structure. To this end we shall minimize the role of interfacial tension^{1,2} as well as intermolecular diffusion³. Therefore, we chose a viscous fluid with zero interfacial tension against water, an aqueous polymer solution of high relative molecular mass polysaccharide for which the mixing time is very slow compared with the time of the experiment.

Since the viscosity difference is the driving force for the instability, a high viscosity is desirable. Hence we use a non-newtonian fluid: the low-shear regions have high viscosity while the shear thinning character of the fluid makes it easy to pump it through the narrow gap of the Hele-Shaw cell without causing

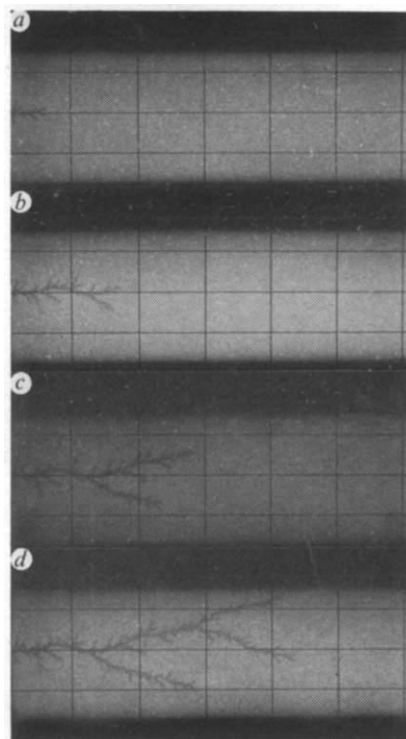


Fig. 1 Typical viscous finger created by water advancing into a Hele-Shaw cell filled with a non-newtonian fluid with zero interfacial tension (experimental conditions described in the text). The time interval between successive photographs is typically 5 s. The grid rectangles have dimensions 3 cm $\times$ 5 cm. The water, dyed with methylene blue, is injected under force from a single inlet on the left wall into an aqueous polymer solution whose viscosity varies between 10^3 and 10^4 cP depending on the water velocity.

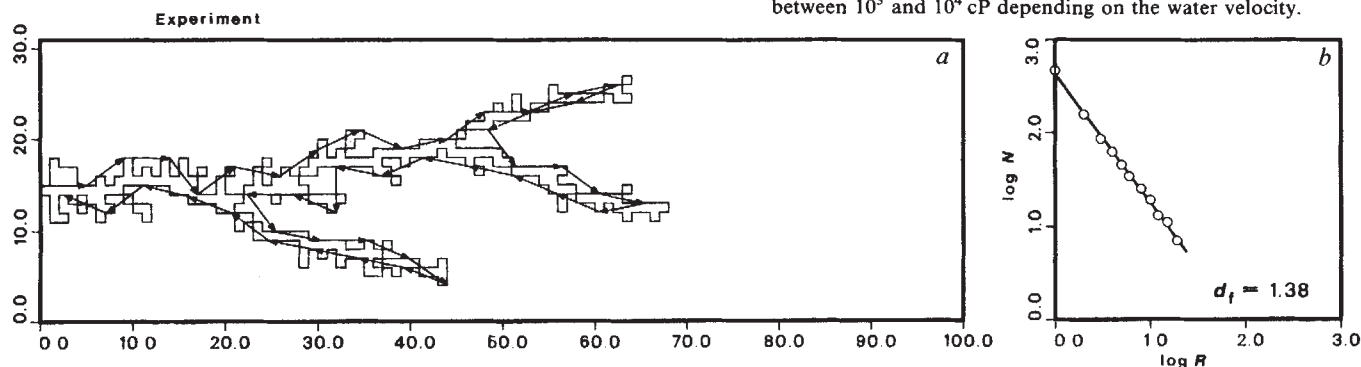


Fig. 2 Schematic illustration of the determination of d_f from experimental data of Fig. 1. *a*, Illustration of how the contour length of the digitized finger is measured with a ruler of length $R = 5$. *b*, The slope of the straight line gives $-d_f$, from which we find $d_f = 1.39$.

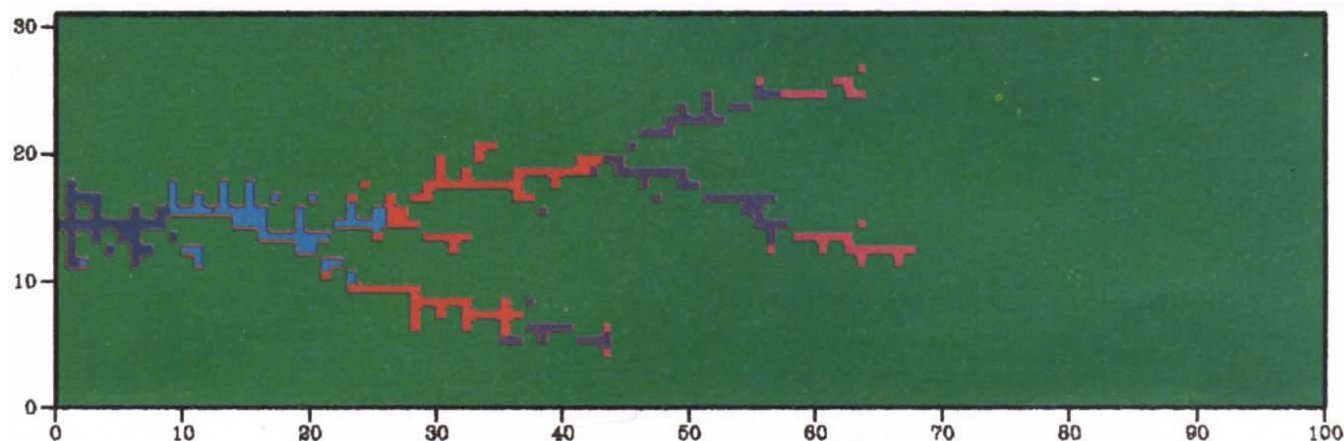


Fig. 3 Digitized representation of the viscous finger of Fig. 1. The colour coding is an indication of the time evolution of the instability, the order being dark blue, light blue, red, violet and pink. The colour coding is for comparison with the model calculations of Fig. 4.

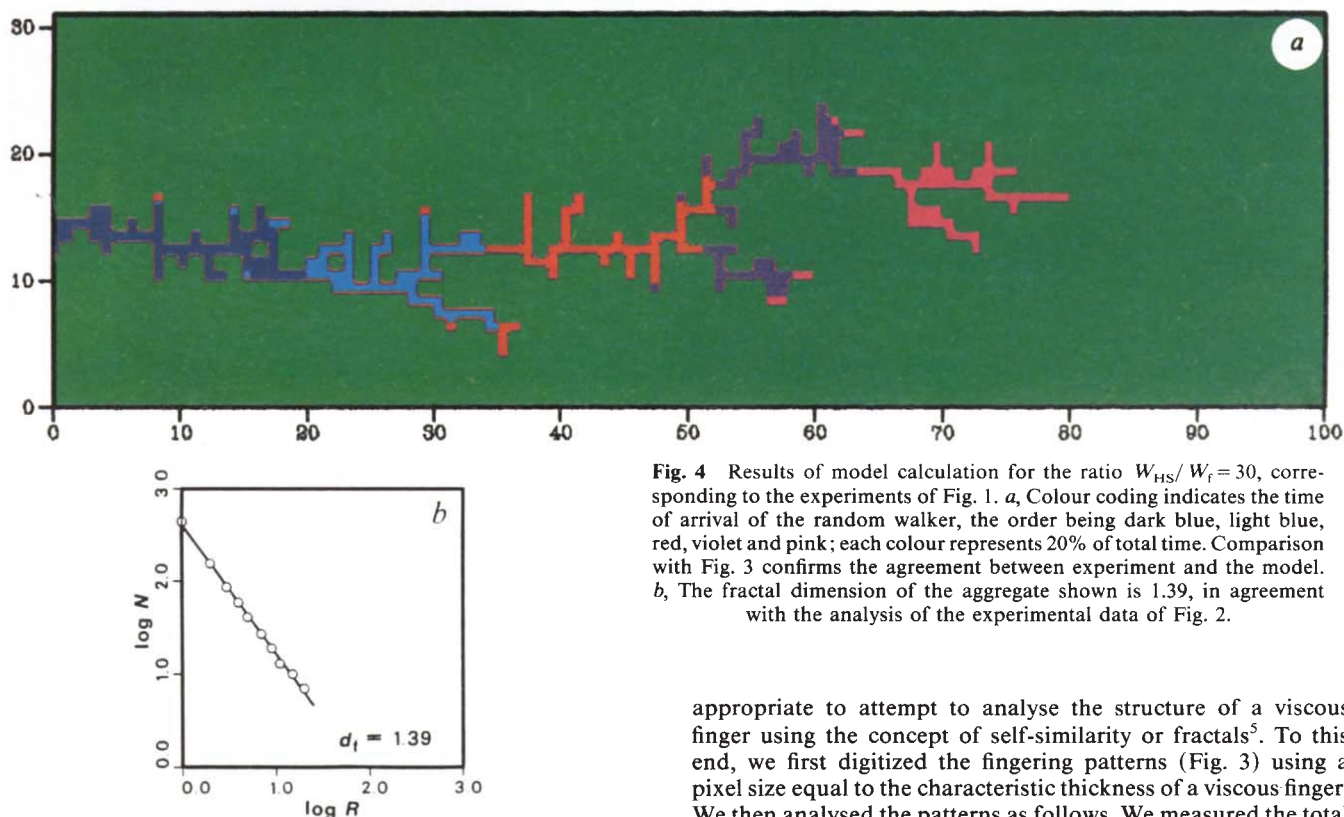


Fig. 4 Results of model calculation for the ratio $W_{HS}/W_f = 30$, corresponding to the experiments of Fig. 1. *a*, Colour coding indicates the time of arrival of the random walker, the order being dark blue, light blue, red, violet and pink; each colour represents 20% of total time. Comparison with Fig. 3 confirms the agreement between experiment and the model. *b*, The fractal dimension of the aggregate shown is 1.39, in agreement with the analysis of the experimental data of Fig. 2.

bending of the plates. In contrast, tests with newtonian fluids were limited to a viscosity of $100\times$ that of water, and steady fine-structured fingering patterns were not observed.

We placed the polymer solution into a perpex Hele-Shaw cell⁸ of thickness 0.5 mm, length 0.9 m and width 0.1 m. Water dyed with methylene blue was injected at one end at a variety of flow rates between 0.4 and 40 ml min⁻¹. We used three different polysaccharides and have varied the polymer concentration from 0.24 to 1.2 wt %. The viscosity of the polymer solution varies between 10^3 and 10^4 cP depending on the water velocity. We have also repeated measurements using a Hele-Shaw cell of thickness 1.0 mm. The resulting viscous finger patterns were random and highly branched (Fig. 1, *a-d*).

Analysis

Visual examination of these patterns reveals the striking presence of structure at many different length scales. Hence it seemed

appropriate to attempt to analyse the structure of a viscous finger using the concept of self-similarity or fractals⁵. To this end, we first digitized the fingering patterns (Fig. 3) using a pixel size equal to the characteristic thickness of a viscous finger. We then analysed the patterns as follows. We measured the total exterior perimeter length using a 'ruler' of length $R=1$, measured in units of a pixel. Then we repeated the same operation for a range of increasing R values up to $R=20$ (Fig. 2*a*). The apparent length of any fractal, in units of R , decreases as R^{-d_f} (Fig. 2*b*)^{5,9}. The d_f of the external perimeter is the same as the d_f of the total mass if loops do not occur on all length scales⁵. We find the quantitative parameter, $d_f = 1.40 \pm 0.04$, which characterizes viscous fingers for a wide range of polysaccharides, polymer concentrations and water flow rates, suggesting that viscous fingers indeed have a reproducible and universal fractal dimension.

As a second method, we placed an imaginary box of edge L on each point of the finger. For each box, we counted the number of points (the total mass) inside the box. As L increases, this mass should scale as L^{d_f} if the finger has a fractal structure¹⁰. We find that Method 2 gives smaller values for d_f than predicted by Method 1. The difference is $\sim 8\%$ and is explained by the fact that a large proportion of the finger points lie on the

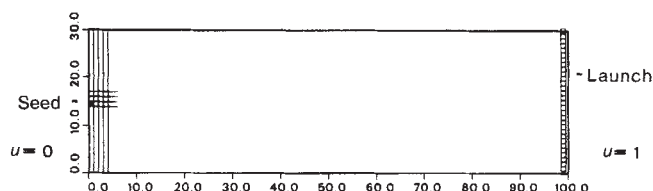


Fig. 5 The basic geometry of the generalized Witten-Sander model used to simulate viscous fingers. Random walkers are released from a randomly chosen point on the right wall, and 'die' if they strike the lateral or end walls.

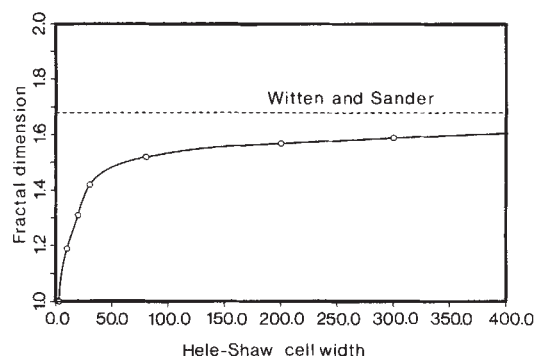


Fig. 6 Dependence on W_{HS} of the apparent value of the fractal dimension, d_f .

perimeter of a particular pattern and boxes centred at these points will cover much unoccupied area outside the structure. For the large-scale numerical simulations discussed below, the number of points on the perimeter is small compared with the total number of points; both methods give identical fractal dimensions.

Statistical model

The mean velocity, v , of a viscous fluid in a Hele-Shaw cell is given by the gradient of a potential $\phi = -(b^2/12\mu)P$, where b is the plate separation, μ is the shear viscosity and P is the fluid pressure¹¹. For incompressible fluids, $\text{div } v = 0$; hence we obtain a Laplace equation for the potential distribution

$$\nabla^2 \phi = 0 \quad (1)$$

If, as in our case, the viscosity ratio between the two fluids is very large, we can approximate the potential in the less viscous fluid to be a constant ($\phi = \phi_0$). At the outlet, ϕ takes on a different value $\phi = \phi_1$.

With these boundary conditions, equation (1) is identical to the Laplace equation describing the probability $u(r, t)$ of a random walker to be at the point r at the time t ^{12,13}. This is similar to diffusion-limited aggregation (DLA)¹³⁻¹⁶, so we propose the following quantitative model for viscous fingering. Place a seed particle at the origin (Fig. 5) and release a random walker from a randomly chosen point on the opposite wall. If the random walker hits the seed, it sticks, whereas if it hits any of the four walls, it 'dies' and a new walker is released. For the simulation, we have chosen our 'Hele-Shaw' cell width $W_{HS} = 30$ (in units of a lattice constant), as the experimental value of $x = W_{HS}/W_f$ is ~ 30 , with W_f the characteristic size of a single finger. Formally, the probability $u(r, t)$ can be mapped onto the fluid potential $\phi(r, t)$ described above, $u(r, t) = (\phi - \phi_0)/(\phi_1 - \phi_0)$. Thus, $\phi - \phi_0$ in the finger corresponds to $u = 0$ in the aggregate, whereas $\phi = \phi_1$ at the outlet maps to $u = 1$ at the source of random walkers.

Two striking features of the actual viscous finger experiments are mirrored by our statistical model. The first of these is the tendency of a growing side branch to die out after a relatively short period of growth. The second is the fact that the entire viscous finger strongly avoids coming even moderately close to the lateral walls of the Hele-Shaw cell. Both effects are seen

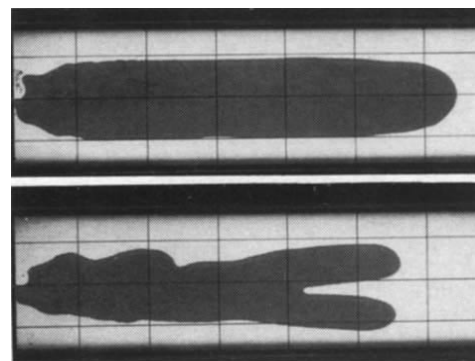


Fig. 7 A viscous finger created by water advancing into a Hele-Shaw cell filled with a newtonian fluid, oil. Two different capillarity numbers are used: *a*, $N_{ca} = 0.08$; *b*, $N_{ca} = 0.16$.

clearly in the model and arise from a common source, 'screening'¹³⁻¹⁶. This screening can be understood as follows. A random walk, statistically speaking, sticks to the tips of the growing aggregate so that the main trunk of the aggregate effectively shields the smaller side fingers. As the lateral walls absorb the random walkers, they act as 'ghost fingers': the viscous finger and the wall together form an essentially impenetrable deep fjord. This can be seen dramatically by the colour coding of Fig. 4, where the last random walkers to arrive, coloured pink, rarely reach the side fingers and never penetrate the deep fjord between the aggregate and the wall.

Calculating d_f as described above, we find $d_f = 1.41 \pm 0.05$ (see Fig. 5), a value remarkably close to the experimental value but considerably different from the value $d_f = 1.68 \pm 0.04$ for DLA¹⁷. Could this difference arise from the geometric constraint of the lateral walls? To test this possibility, we made additional simulations for a range of values of x . We find (Fig. 6) that the apparent value of d_f increases smoothly from $d_f \approx 1$ (for $x \approx 1$) to $d_f \approx 1.60$ (for $x \approx 10^3$). To check this prediction, we also performed experiments for a Hele-Shaw cell half as wide ($x = 15$) and find that $d_f = 1.12 \pm 0.04$, consistent with the predicted value of Fig. 6. What is the source of this apparent dependence of d_f on W_{HS} ? At first sight, one might be tempted by an analogy with a growing polymer chain in the same confining geometry; it takes on the bulk value of d_f until it has grown sufficiently to sense the presence of the lateral walls. However, our problem is quite different, since the lateral walls strongly influence even the initial stages of finger growth because the incoming random walks are strongly influenced by the absorbing lateral and end walls. Hence the small fingers cannot capture enough mass to achieve the full DLA structure (this fact becomes especially clear if we consider very small values of W_{HS}). So while for the polymer problem d_f crosses over directly from its bulk value to its ultimate value of $d_f = 1.0$, for the Hele-Shaw fingering problem d_f never takes on its bulk value but instead takes on a smaller value that depends monotonically on W_{HS} (Fig. 6), crossing over ultimately to $d_f = 1$ when $R \gg W_{HS}$.

Discussion

We consider here the relation of the present experiments to previous work on viscous fingering. The classic Saffman-Taylor work did not produce ramified fractal structures. To obtain fine-structured instability patterns for an immiscible fluid system, the critical wavelength λ_c of a perturbation^{2,18}, defined as $\lambda_c = k[b^2/(12N_{ca})]^{1/2}$ has to be much smaller than W_{HS} . N_{ca} is the capillarity number, defined as $N_{ca} = \mu v/\sigma$ (σ is the interfacial tension) and corresponding to the ratio of viscous forces to capillarity forces: k is a dimensionless parameter that depends on the contact angle between the two fluids and the plates (wettability)¹⁸. We have also performed Saffman-Taylor type experiments with an immiscible newtonian oil-water system with varying flow velocity v , and Fig. 7 indicates that a change

in the flow condition from $N_{ca} = 0.08$ to 0.16 introduces a bifurcation into fingers when the finger thickness exceeds twice λ_c ¹⁹. A continuation of this process would presumably create smaller and smaller fingers with a tree-like structure, but would require a further increase in the capillarity number or a decrease of the plate distance b . We cannot test the possibility that newtonian fluids with very low interfacial tension and negligible intermolecular diffusion but with a very high viscosity ratio, might give rise to similar fractal instability patterns, as our experiments were limited by the finite strength of the cell. By using water and a non-newtonian water-based fluid, we obtained a flow with an infinite capillarity number and a high viscosity ratio in regions where shear stresses are low. We believe both aspects are important and explain the difference from the Saffman-Taylor results.

Our present findings can be applied to the basic physics underlying petroleum science^{4,20}. In the traditional '5-spot well configuration', four production wells are drilled at the corners of a large square and CO₂ (or a low viscosity fluid with a very low interfacial tension with oil) is then pumped into a fifth injection well in the centre. The CO₂ plays the role of the water in our model experiments, whereas the oil is analogous to the polymer solution. Large-scale viscous fingering phenomena not bounded by the walls of a Hele-Shaw cell occur and drastically reduce the efficiency of the oil recovery. The observed viscous fingers are quasi-two-dimensional as the thickness of the rock formation is typically several orders of magnitude less than the separation of the injection and production wells. It is therefore customary to assume that the patterns observed in the quasi-two-dimensional Hele-Shaw cells are relevant to the observed macroscopic phenomena. The similarity in the mathematical descrip-

tion of flow through porous media and the Hele-Shaw cell have been observed previously¹. Thus, our experiments and model calculations strongly suggest that the viscous fingers observed in a 5-spot pattern are rich geometrical structures possessing a scale-invariant symmetry and quantitatively characterized by a fractal dimensionality.

Conclusion

There is no complete theory of miscible viscous fingering, perhaps because there was no quantitative parameter to characterize viscous fingers. Here, we find that viscous fingers are fractals and that their many different random structures have the identical value of the fractal dimensionality. This suggests that the physical basis of the viscous fingering instability is analogous to that of other second-order phase transitions that obey the same sort of scale invariant symmetry found here for viscous fingers²¹. Recent work supports our point of view: quasi-two-dimensional dielectric breakdown seems to exhibit the same sort of fractal structure that we find for the viscous fingering instability²². That these two breakdown phenomena should be related is plausible in light of the parallels in the equations describing them²³.

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Note added in proof: Since acceptance of this article, we have learned of independent work on viscous fingering, both from the experimental (ref. 24, and L. M. Sander and E. Ben-Jacob, personal communication) and theoretical^{25,26} points of view.

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LETTERS TO NATURE

A very bright water vapour maser source in the galaxy NGC3079

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The 6₁₆-5₂₃ transition of water vapour at 22.235080 GHz has been found in our Galaxy to be associated with regions of young star formation^{1,2}. H₂O maser emission was detected initially in an external galaxy by Churchwell *et al.*³. To extend the range of detected properties of 'star burst' galaxies (galaxies showing an enhanced star formation from their optical spectra) we undertook an initial survey for H₂O maser emission in this type of galaxy, but with negative results. Here we report on the subsequent extension of this survey to spiral galaxies displaying continuum radio emission, which is also an indication of star formation activity. We have detected the most luminous H₂O maser source yet reported, ~500 L₀, in the galaxy NGC3079. OH absorption was also detected in this galaxy and we suggest that maser amplification of the nuclear radio source causes the observable H₂O emission.

The 30 galaxies for the survey reported here were selected from lists^{4,5} of bright spiral galaxies containing radio sources having both compact and extended components. The observations were carried out in March, April and May 1984 using the 37-m radio telescope of Haystack Observatory, equipped with a maser receiver and a 1,024 channel digital auto-correlator. The system temperature is ~90 K and the bandwidths 20 and 40 MHz, providing resolutions of 0.8 and 3.2 km s⁻¹, respectively, at the rest frequency of 22.235080 GHz. Each source was observed in the total power mode for 3 h; the antenna beam is 90 arcs and covers only the central part of each galaxy.

In Fig. 1 is a spectrum for NGC3079 showing a strong central water vapour feature of ~5 Jy in size and having a width of ~10 km s⁻¹; in addition, there are weaker emission features of <1 Jy extending out to velocities of ±80 km s⁻¹ from the central feature. The strongest feature in NGC3079 is blueshifted by ~200 km s⁻¹ from the systemic velocity of the galaxy of 1,171 km s⁻¹ (ref. 6). In Fig. 2 is a spectrum (taken on the NRAO 300-ft telescope in May 1984) of the absorption by the OH radical at 1667.358 MHz in the radio continuum of NGC3079 (see also B. Turner, I. Kazes and L. J. Rickard, personal communication). This spectrum shows a deep absorption feature ($N_{OH}/T_{EX} = 7.6 \times 10^{15} \text{ cm}^{-2} \text{ K}^{-1}$) at lower velocities than the systemic velocity of the galaxy. The velocity of the H₂O maser emission is indicated lying on the blueshifted edge of the OH absorption, implying that the H₂O maser source is foreground

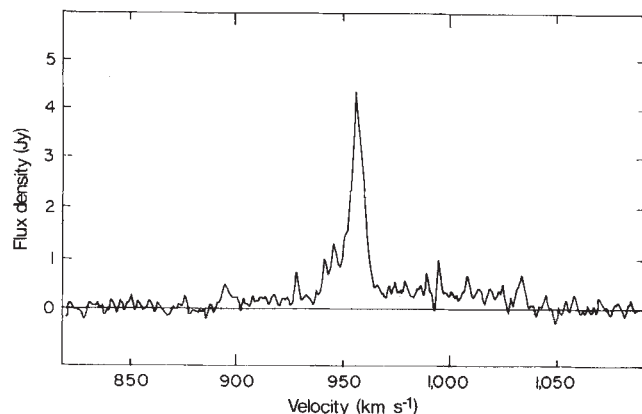


Fig. 1 A spectrum of NGC3079. The rest frequency is 22.235080 GHz and the resolution is 1.3 km s^{-1} . Velocity is given by $c\Delta\lambda/\lambda_0$, where c is the velocity of light and λ , the wavelength.

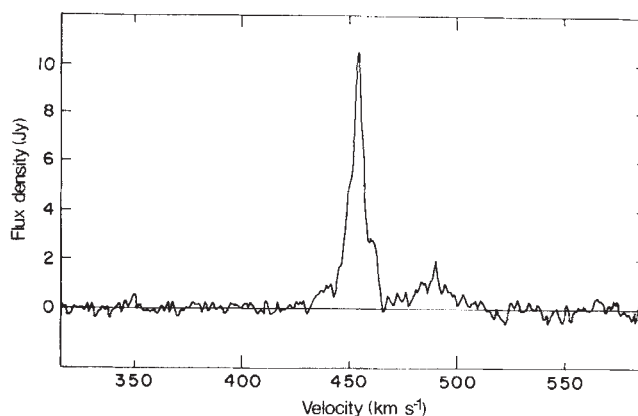


Fig. 3 A spectrum of NGC4258. The resolution and rest frequency are as in Fig. 1.

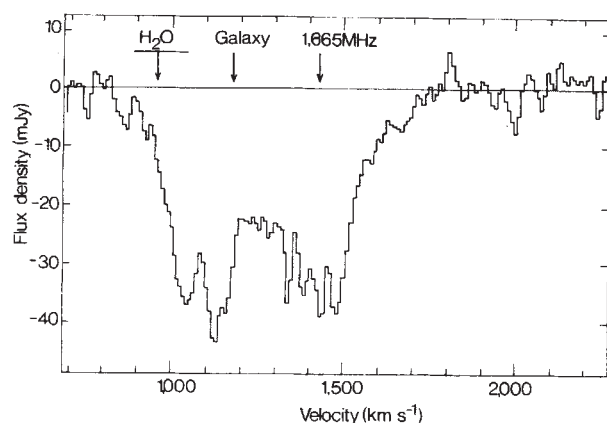


Fig. 2 An OH spectrum of NGC3079 taken on the NRAO 300 ft antenna in Greenbank, West Virginia. The rest frequency used is 1,667.358 MHz. Also indicated on the plot are the velocities of the H_2O maser source and the 1,665 MHz absorption feature. The 1,665 and 1,667-MHz absorption features are slightly blended. The galaxy velocity is also indicated. This absorption feature has been independently detected by Turner, Kazes and Rickard (personal communication).

Table 1 Sources in which H_2O was not detected

Source	S_{lim} ($3 \times \text{r.m.s.}$) (Jy)	Velocity (km s^{-1})
NGC520	0.5	2,100
NGC660	0.4	856
NGC891	0.4	72
NGC972	0.7	1,555
NGC1068	1.0	1,135
NGC1073	0.4	1,212
NGC1087	0.3	1,824
NGC1569	0.4	-42
NGC2146	0.5	812
NGC2276	0.6	2,391
NGC2903	1.0	644
NGC3034	0.7	186
NGC3628	0.4	842
NGC3690	0.12	3,100
NGC3310	0.4	1,026
NGC3504	0.5	1,526
NGC3556	0.4	697
NGC4051	1.2	658
NGC4102	1.1	897
NGC4151	1.1	457
NGC4157	0.5	771
NGC4303	1.5	1,671
NGC4321	0.7	1,617
NGC4565	0.5	1,183
NGC4656	1.1	755
NGC4631	0.6	626
NGC4490	0.5	570
NGC5055	0.6	520
NGC6946	0.5	80
NGC7331	0.4	950
NGC7714/5	0.7	2,833

to the radio source. It is likely to be close to the nucleus and part of an outflow of gas from the galaxy nucleus.

The H_2O spectrum of NGC4258 is given in Fig. 3 and shows a significant change from that given by Claussen, Heiligman and Lo⁷ for January 1983 in that a feature at 455 km s^{-1} has risen to a level of $\sim 10 \text{ Jy}$. Spectra taken in August 1984 (G. Garay, J. M. Moran and A.D.H., unpublished results) show this feature to have declined to $\sim 5 \text{ Jy}$. As the weak feature at $\sim 490 \text{ km s}^{-1}$ is approximately the same amplitude of $\sim 1.5 \text{ Jy}$ in the January 1983 spectrum as in April 1984, it may be constant, with the difference in shape being attributable possibly to the poorer signal/noise ratio of the later spectrum. The linear polarization of the brightest velocity features in the maser sources in NGC3079 and NGC4258 was measured (G. Garay, J. M. Moran and A.D.H., unpublished results) using the Haystack antenna and found to be $< 6\%$ for NGC3079 and $< 2\%$ for NGC4258. The non-detections of galaxies are listed in Table 1 together with their upper limits.

The maser source in NGC3079 is the most luminous maser source yet detected having a peak isotropic luminosity in June 1984 of $\sim 500 L_0$, assuming a distance to NGC3079 of 16.5 Mpc ($H_0 = 75 \text{ km s}^{-1} \text{ Mpc}^{-1}$). NGC3079 and the previously detected galaxies NGC4258 ($130 L_0$, this observation) NGC1068⁷ ($350 L_0$), NGC4945⁸ ($240 L_0$)⁹ and the Circinus Galaxy¹⁰ ($37 L_0$), form a class of galaxies that contain highly luminous water vapour maser emission. For comparison, in our own

Galaxy the strongest source known, W49N, has a luminosity of $< 1 L_0$ and all other extragalactic water vapour masers detected thus far are $< L_0$.

One interpretation⁷ for the high luminosities of the H_2O maser sources in these galaxies is that they could indicate a burst of star formation of 10^5 – 10^6 stars assuming that the maser emission is the sum of a number of individual sources and considering that the H_2O luminosity of an average star formation region in our galaxy is $10^{-3} L_0$. This is similar to the burst of star formation of $\sim 10^8$ stars that would be predicted from a comparison of luminosities of the bright OH maser emission detected by Baan, Wood and Haschick¹¹ in the galaxy IC4553 (Arp 220).

An alternative explanation for these bright maser sources may lie in the supposition that we are seeing low-gain maser amplification of the background continuum radio source. A salient characteristic of these five galaxies is the presence of nuclear radio sources in all cases^{8,9}. In the example of the OH megamaser IC4553, the emission is found to be exactly superimposed

on the nuclear radio source, which is resolved into three components; relatively low-grain maser amplification of the background source is suggested as being the mechanism for producing the intense OH-maser emission (ref. 12 and R. P. Norris, W.A.B., A.D.H., R. S. Booth and P. J. Diamond, in preparation), even though the masing region in front of the central component may be partially saturated. This indeed may also be the mechanism producing the intense H₂O emission in the above mentioned galaxies. Recent Very Large Array observations with a resolution of 2.5 arc s indicate that the brightest feature in NGC3079 is superimposed on the compact nuclear radio source. Furthermore M. J. Claussen *et al.* (personal communication) report that the strong maser feature in NGC1068 is coincident with one of the three radio-continuum knots in the nucleus of the galaxy and that the H₂O features in NGC4258 are coincident with the nuclear radio source in this galaxy. These positional coincidences may be characteristic of the emission mechanism responsible for the H₂O lines. The narrower H₂O velocity features, $\sim 10 \text{ km s}^{-1}$ for the main features seen in these galaxies, as compared with 108 km s^{-1} in IC4553, would indicate higher-gain amplification by fewer H₂O masing regions than in the OH example and a smaller size for the H₂O sources. The brightness temperature of the H₂O features in NGC3079 will determine whether the amplification hypothesis is applicable in this case. However, a molecular region with an inverted H₂O population will amplify radiation from a background continuum source until the maser reaches saturation and is observable; without the background source the maser may be weaker or not observable.

Maser amplification of the background continuum would not eliminate the necessity for highly luminous pump sources for the masing regions and it is possible to postulate that bursts of star formation supply the energy for these maser sources. All the powerful extragalactic masers are associated with galaxies having both a high infrared luminosity, which is thought to indicate a large number of hot massive stars enshrouded by dust and signs of nuclear activity. Galactic H₂O maser sources are thought to be pumped collisionally¹³, but as these extragalactic amplifying masers could be only partially saturated, the pump source may be radiative requiring only a modest burst of star formation ($< 10^3$ stars). Pump models as suggested by Strelitzkij¹⁴ could present further alternatives to explain the luminous maser emission.

It also seems pertinent that three of the five luminous masing galaxies are edge-on galaxies (NGC1068 and the Circinus galaxy being the two exceptions), which raises the molecular column density and thus the probability of having H₂O masing regions on the line of sight to the nuclear radio source. The presence of OH absorption in two of the galaxies (NGC3079 (see Fig. 3 and B. Turner, I. Kazes and L. J. Rickard, personal communication) and NGC4945; ref. 15) indicates the presence along the line of sight of dense molecular clouds. The relatively large number of non-detections of strong H₂O maser sources in galaxies having radio continua would imply that the presence of a nuclear radio source is a necessary, but not sufficient, condition for this maser emission. Thus, it seems plausible that these superluminous extragalactic maser sources may simply comprise a number of individual masing regions which are foreground to the nuclear radio source.

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Periodicity in the BL Lac object OJ287

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Short-term variations in luminosity provide the best upper limits for the sizes of active galactic nuclei. Periodicity provides further information on the geometrical, dynamical and physical models of these regions. The best candidates for the study of such variations are the so-called blazars (optically violently variable (OVV) objects and BL Lac objects). Our photometric observations of OJ287 reveal periodic variability in timescales of a few minutes. We conclude that our results are compatible with the presence of 'hotspots' in a thick accretion disk spiraling into a Kerr's supermassive black hole and that the central source is seen nearly pole-on.

The highly variable nature of OJ287 at visible, radio and infrared wavelengths is well established^{1,2}. However, the possibility of periodic light changes in this object has created some controversy³⁻⁵. Recent observations⁶ at radio wavelengths (22 and 37 GHz) seem to indicate that OJ287 could vary regularly with a periodicity of 15.7 min. We have undertaken an observational program to search for regular time variations in the light of blazars, looking particularly at OJ287 as a promising candidate. The light curve of OJ287 has been observed with a 1-s time resolution, during eight night runs collected between January and March 1983, with two additional runs in February 1984. Table 1 summarizes the dates and duration of the observations. The observations were carried out using a high-speed two-channel photometer, developed by Nather⁷, attached to the 1-m telescope at Tonantzintla Observatory. To maximize the photon counting rate, the observations were carried out without filter, using the full spectral response of the photomultiplier (RCA 8850) which yields an instrumental effective wavelength similar to that of Johnson's B band. This two-channel photometer allows simultaneous observations to be made of comparison sources, so enabling fluctuations in atmospheric transmission and extinction to be removed from the observed light curves. From photometry done through several apertures, we find that 99% of the light was contained within 15 arc s. To eliminate instrumental modulation, we chose a 29-arc s diaphragm. We applied the same observational and reduction techniques to stellar objects to determine the instrumental effects;

Table 1 Observational data

Date (UT)	Duration (s)
17.2722 January 1983	3,200
17.3104 January 1983	3,200
16.0674 February 1983	5,000
17.2681 February 1983	3,700
18.2556 February 1983	4,900
10.1146 March 1983	12,700
11.0847 March 1983	15,500
12.2201 March 1983	2,300
25.2688 February 1984	8,400
26.1257 February 1984	13,100

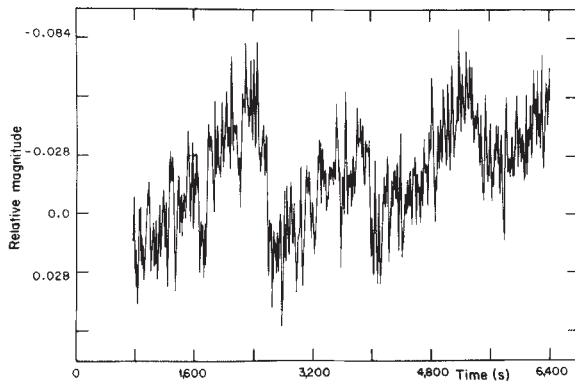


Fig. 1 Observed light curve for the night of 10 March 1983. $t = 0$ corresponds to 2h 51.7 min (UT).

none were found that affected our conclusion regarding the periodicity of OJ287.

A typical corrected light curve of OJ287 during the 1983 active period is shown in Fig. 1. The periodicity of the light variations indicates a period of 23 min. Furthermore, a fast Fourier transform analysis of the time series has been performed on the individual runs and on all the observations carried out in 1983. For the latter, the Deeming⁸ algorithm was used on zeroed and edge-smoothed data. In every case, we find significant power at the frequencies corresponding to the periods listed in Table 2 (see also Fig. 2). Our analysis implies that the detected light variations are coherent over at least the two months in 1983 during which the observations were carried out. The active period of OJ287 could have lasted a year, or be present again at the beginning of 1984, when the observations were repeated and the same results obtained. Note that the most evident frequency present in our data set is roughly double the frequency detected during the 1972 active period of OJ287.

From the Fourier analysis of individual nights, a little power at the fundamental frequency of the gear's periodicity was seen, corresponding to a period of 138 s. The amplitude for the 23-min period, the most prominent one during the observation, was 0.030 ± 0.005 m. From the apparent magnitude of the object ($m_v \sim 12$) at those times and a redshift $z = 0.301$ (ref. 9), we obtain 4×10^{45} erg s⁻¹ for the visible luminosity of the modulated component.

Note that at the epoch of our observations (1983–84), OJ287 was at maximum brightness ($m_v \sim 12$), which was also the case 10 years ago when Visvanathan and Elliot³ reported light variations with a 40-min period, which were confirmed a year later by Frohlich *et al.*⁴. On careful examination of the light curve in ref. 3, however, a period of ~ 20 min cannot be excluded.

One way in which short-period variations could be produced involves the pulsational modes of self-gravitating accretion disks around supermassive black holes¹⁰. Grauer¹¹ suggested that quasiperiodic optical variations of the OVV 4C29.45 over time scales ~ 20 min could be interpreted in terms of this idea. However, this model has to be worked out more carefully as the driving force behind those oscillations has not been analysed, nor is there any obvious reason to pick out a specific annulus of the disk that dominates the pulsations. A more sensible way

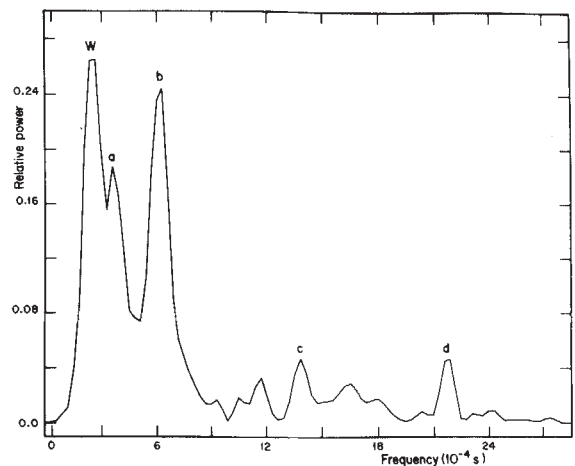


Fig. 2 Power spectrum derived from Fourier analysis with the Deeming algorithm for unevenly spaced points, applied to the 1983 data. Feature W corresponds to the observational time window. Features a, b, c and d are produced by the intrinsic shape of the light curve of OJ287. The corresponding periods are listed in Table 2.

of explaining short-period regular variations is to assume that the accretion disk contains one or various 'hotspots' which are spiralling into a black hole¹². For a Schwarzschild hole, thin accretion disks have their innermost stable orbit at $6GM/c$, and thick disks reduce this value at $4GM/c$ (ref. 13). The radius of the event horizon of a Kerr black hole of mass M and angular momentum parameter a is (P. J. Wiita, preprint)

$$r = 1.48 \times 10^5 (1 + \sqrt{1 - a^2}) (M/M_\odot) \text{ cm} \quad (1)$$

In the case of an extreme Kerr black hole ($a = 1$), both the innermost bound and stable orbits coincide at $r = GM/c$. Thus a periodic phenomenon could exist down to timescales of

$$P = 2\pi r/c \approx 3.1 \times 10^{-5} (M/M_\odot) \quad (2)$$

This expression yields a mass for the central black hole of $3 \times 10^7 M/M_\odot$ for a 23-min period and $6 \times 10^7 M/M_\odot$ for the 40-min one. These masses lead to Eddington luminosities of 5×10^{45} and 1×10^{46} erg s⁻¹ respectively. These numbers are too small to explain the object's bolometric luminosity (we recall that at maximum light, $L_v \sim 10^{47}$ erg s⁻¹). However, thick accretion disk models allow for actual luminosities that exceed L_E (ref. 14). Furthermore, one must still consider the effects of directed relativistic outflow (beaming)¹⁵. For a bulk relativistic Lorentz factor γ , the observed luminosity is enhanced by a factor of γ^2 and the total mass-energy required (for a fixed efficiency in the comoving frame) is reduced by a factor γ^4 .

We conclude that the model that can explain our observations is one in which 'hotspots' in a thick accretion disk are spiralling down to a Kerr's supermassive black hole and are being periodically occulted by the disk itself. To have a beaming effect and to see the hotspots at the innermost part of the source, we must be viewing it almost pole-on (within $\sim 11^\circ$ of the rotation axis of the disk according to Sikora¹⁶) and the Thompson scattering depth τ_T , should be < 1 , which is likely to be the case for highly polarized objects (P. J. Wiita, preprint). The conflict between the real and observed luminosities can be resolved if an apparent luminosity of $\sim 10 L_E$ is produced by thick disk accretion coupled with bulk relativistic motions with $\gamma \sim 2$.

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Table 2 Observed periods

Fast Fourier transform (s)	Deeming (s)	Feature*
2,500†, 2,710	2,532	a
1,355	1,370	b
942‡	—	—
—	721	c
455	459	d

* See Fig. 2. † See ref. 4. ‡ See ref. 6.

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A 15.7-min periodicity in OJ287

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The BL Lac-type object OJ287, with a redshift $z = 0.306$ and a luminosity exceeding $10^{47} \text{ erg s}^{-1}$ at maximum light, is among the brightest and most violently variable objects of its class. Rapid variations, with timescales of a few hours or less, have been reported over a wide range of wavelengths^{1,2} and also in polarization³, imposing strong constraints on the size and dynamics of the source^{4,5}. The detection of periodic variations in OJ287, or in any other compact extragalactic source, would be even more important, as these could be related to rotation or orbital motion in the source. So far the only searches sensitive to periodic variations shorter than 1 h are the photometric observations of Visvanathan and Elliot⁶, Frohlich *et al.*⁷ and Kiplinger⁸, in which a 40-min period in OJ287 was respectively found, confirmed and not detected. We report here the detection of a 15.7-min periodic flux variation in OJ287 in April 1981 at 37 GHz. The source has been subsequently monitored for 265 h at 22 and 37 GHz, as well as in optical photometry, and the same period has been seen in simultaneous observations. The peak–peak amplitude of the variation is 1–10% of the total intensity. Other periods may also exist.

In the Turku–Otaniemi quasar variability monitoring programme⁹, 20 quasar-type objects are observed for a period of, in the mean, one week each month with the 13.7-m radio telescope of the Helsinki University of Technology at Metsähovi, Finland. Each source is observed for a period of 1–2 h. During the measurement, a computer printout for every single integration segment (usually 50 s) is provided for control of the observation. On inspecting the printouts, we found that OJ287 was behaving differently from other sources and that an approximately 15-min periodicity was at times clearly visible even among the large noise of single integrations¹⁰. Subsequently, we observed OJ287 on several nights to ascertain whether periodic variations are really present.

The radio observations were made between April 1981 and May 1983. OJ287 was monitored for a total 265 h during 47 separate observing runs. We have used different observational procedures and integration times to check that the periodicities are not artefacts of our data-collecting methods or the spacing of observations. (Details of the observational procedures are presented elsewhere⁹.) A typical run lasted about 6 h, with one data point (~ 50 s of integration time) produced every 2 min.

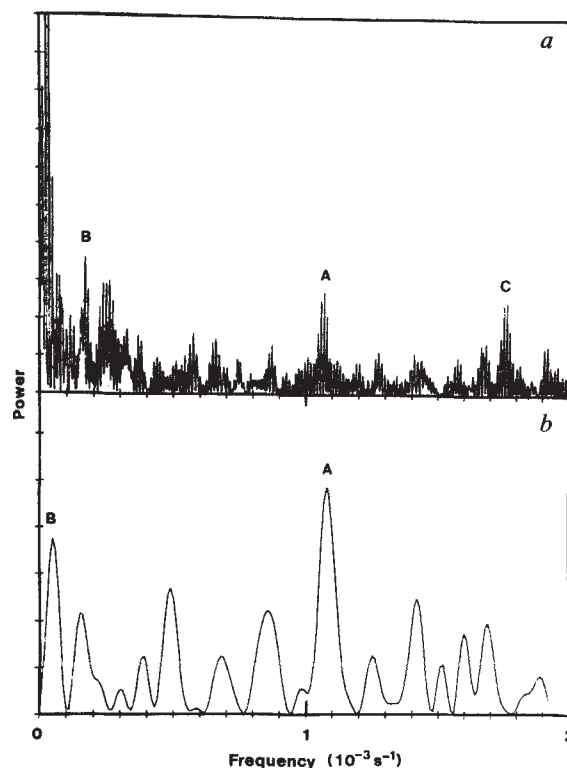


Fig. 1 *a*, Power spectrum of the 37-GHz observations with the Metsähovi radio telescope on 24 April 1981. A, the 15.7-min peak; B, peak caused by the data window. *b*, Combined power spectrum of the 22-GHz observations with the Metsähovi radio telescope on 5–7 March 1982. A, the 15.7-min peak; B, peaks caused by the data window and long-time changes in the signal level; C, peaks caused by instrumental effects.

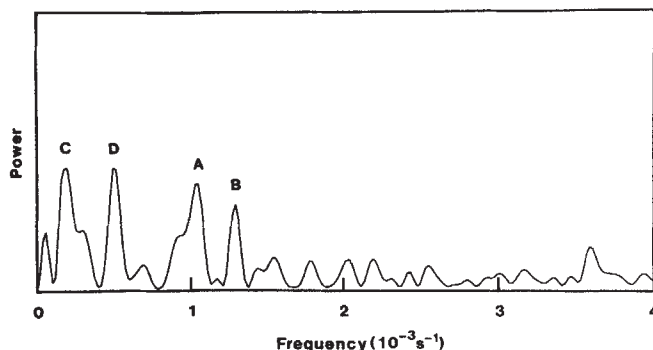


Fig. 2 Power spectrum of the 22-GHz observations with the NRAO 140-ft telescope on 23 May 1983. A, the 15.7-min peak; B, the 13.0-min peak; C, peak caused by the data window; D, a spurious peak, not seen in the Metsähovi power spectrum from the same day.

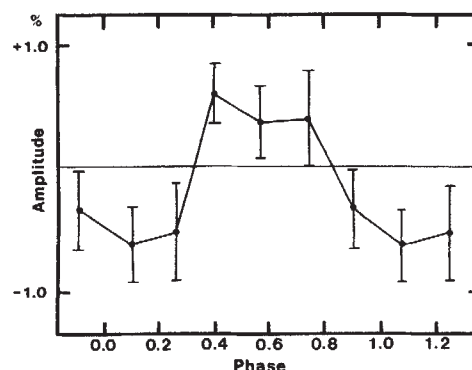


Fig. 3 The mean light curve of the 15.7-min variation from the NRAO observations. Error bars, $\pm 1\sigma$.

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Thus, we had $n \sim 200$ points per run, with equal or unequal spacing (depending on the observing method) and some gaps attributable to calibration and optical depth measurements. The data for each run were then analysed by the Fourier technique of Deeming¹¹. Because of the noise and data spacing, many peaks are apparent on the spectrum of a typical run. Among these peaks, one corresponding to a 15.7-min period is clearly visible on many nights and is shown in Fig. 1a for the run on 24 April 1981.

For a closer inspection of the observations, we have used the periodogram technique of Warner and Robinson¹², which is better suited for noisy data and non-sinusoidal variations. On nights where Fourier analysis indicates the presence of a clear 15.7-min variation, the periodogram technique helps to confirm the period, as well as giving the phase and the light curve of the variations.

On several occasions we were able to observe OJ287 on several consecutive nights. Although the long gaps between runs cause strong aliasing peaks, these can be identified by calculating the spectral window. If the 15.7-min variation is real, we would expect it to stand out more clearly in the combined data, whereas spurious peaks due to noise, unable to keep either phase or period from night to night, would be suppressed. Figure 1b shows the results of Fourier analysis for the combined runs of 5–7 March 1982 (595 observing points). Periodogram analysis gives a period of 943 s, in accordance with Fourier analysis. A comparison with similar data from combined runs, May 1981 onwards, indicates that the period may be constant to an accuracy of 1 s yr^{-1} .

To identify possible instrumental variations, we have made similar observations of a number of other sources and pure background noise, analysing these using the same techniques. Certain periodicities, for example, the peaks marked C in Fig. 1b, are caused by the radome and the variations in the electric current and are identified in most observing runs. Although no observing run is ever exactly repeatable and the periodicity could perhaps have been caused by some unknown mechanism, the most probable explanation is that the 15.7-min periodicity is intrinsic to OJ287, as we have not detected it in any of the other sources.

To ascertain whether the periodicity would be detectable with other instruments, simultaneous measurements were made on 26 February 1982 using the radio telescope at Metsähovi operating on 22 GHz, a double-beam chopping photometer using the 60-cm telescope of Metsähovi Observatory and using a similar photometer coupled to the 60-cm telescope at Turku Observatory, about 150 km west of Metsähovi. (The construction and operating principle of the photometers have been described by Pirola¹³.) A standard observing procedure, with comparison stars measured at regular intervals, was followed. The measurements were made through the B filter. At the time of the observations, OJ287 was rather faint, with B (magnitude) = 14.6 mag, and the noise level of optical observations was rather high. The power spectra of all three runs were calculated and compared with each other and all three instruments showed a peak near the 15.7-min period: the radio telescope at $945 \pm 15 \text{ s}$, the Metsähovi photometer at $980 \pm 35 \text{ s}$ and the Turku photometer at $935 \pm 35 \text{ s}$, with the formal error limits derived from half-widths of the peaks.

To check for a coincidence, we further investigated all the peaks visible in each spectrum using the periodogram technique. In the case of the optical observations, the only periodicities found to have the same phase (within observational error) were those corresponding to the 15.7-min peak. In addition, the radio observations gave a similar light curve, but out-of-phase, and the amplitude of the variations seems to be the same at both wavelengths.

OJ287 was again observed with the 140-ft telescope of the National Radio Astronomy Observatory (NRAO) on 23 May 1983. The effective single integration times were 27 s and the total integration lasted 7.6 h, producing 330 observing points in all. Figure 2 shows the resulting power spectrum and Fig. 3

gives the mean light curve of the 15.7-min variation together with 2σ error bars. In addition to the 15.7-min power spectrum peak, strong peaks are visible at 33 min and 13 min. Both the 15.7-min peak and the 13.0-min peak were outstanding in the Metsähovi 37-GHz data (298 points over 6 h), also taken on 23 May 1983, but the 33-min peak was not observed.

Consequently, the 13-min periodic component, which has appeared on several occasions, has also attracted our attention. In addition, others¹⁴ have observed a strong periodicity at 23 min between January and March 1983 in OJ287. Whether these periods are as durable as the 15.7-min period remains to be seen. Even in the case of the 15.7-min period, there are strong fluctuations in the amplitude, of $>15\%$ to $\sim 1\%$. An interesting parallel may be provided by the infrared observations of Wolstencroft *et al.*², where rapid, but presumably nonperiodic, variability was seen on only one night out of a total of 12, accompanied by a change in the shape of the infrared continuum. Simultaneous observations with different instruments are the only way to obtain definite proof for the existence of variations of this kind.

If only a single periodic component is confirmed, one may identify it with the rotation period of a single supermassive body^{15–17}. However, if the evidence for many periodic components is sustained, then it is more probable that we are observing keplerian motion around a single dominant body with many different orbital radii, analogous to the modulation of the radio emission of Jupiter by its innermost moons. In any case, short periods such as 15.7 min indicate a relatively small mass for the central body ($\leq 10^7 M_\odot$) and a problem with the high luminosity of the quasar^{4,5}.

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Influences of precipitation changes and direct CO₂ effects on streamflow

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Increasing atmospheric carbon dioxide concentrations are expected¹ to cause major changes in the world's climate over the next 50–100 yr. The impact of such changes on water resources, through changing precipitation and evaporation, will, however, be complicated by the direct effects of increasing CO₂ on vegetation. In controlled environment experiments, higher CO₂ levels cause the stomata of plants to close down, decreasing their rate of transpiration and increasing their water use efficiency². Reduced evapotranspiration would make more water available as runoff and could

tend to offset the effects of any CO₂-induced reductions in precipitation or enhance the effects of precipitation increases. We consider here, in a simple but revealing analysis, the relative sensitivity of runoff to these two processes, changes in precipitation and changes in evapotranspiration. We show that, for low runoff ratios, small changes in precipitation may cause large changes in runoff. The magnitude and direction of these changes is, however, strongly dependent on the magnitude of the direct CO₂ effect on plant evapotranspiration.

Although much has been published on the climatic effects of increasing CO₂, few studies have considered the impact on water resources. Revelle and Waggoner³ used empirical relationships to study the effect on water supplies in the western United States of a 10% reduction in precipitation and a 2 °C warming. They suggested that there could be large reductions in river flow. Rind and Lebedeff⁴ used the results of a general circulation model (GCM) to examine possible runoff changes in the United States in a high-CO₂ world. Their results differed from those of Revelle and Waggoner, partly because of differences between the model-generated changes in climate and the scenario assumed in the earlier work, and partly because GCM-generated runoff data cannot be directly equated with measured riverflow (see ref. 5). Neither of these studies considered the direct effects of CO₂ on vegetation. Following Aston⁶ (who used a distributed deterministic process model to simulate the effects of changed stomatal resistance on streamflow), Idso and Brazel⁷ added the direct CO₂ effects associated with a CO₂ doubling to Revelle and Waggoner's analysis. The 40–70% reductions in streamflow in the absence of these direct effects became 40–60% increases when they were included. Such increases are of the same size as those predicted by Aston for catchments in eastern Australia. But how representative are these results?

If non-evaporative losses are small the mean annual water balance of a river catchment is given by

$$R = P - E \quad (1)$$

where R is runoff (here synonymous with streamflow), P is precipitation and E is evapotranspiration. The runoff ratio, γ_0 , is defined by

$$R_0 = \gamma_0 P_0 \quad (2)$$

where subscript zero is used to denote present-day values. To estimate changes in runoff due to changes in precipitation and evapotranspiration we denote the change in precipitation (from P_0 to P_1) due to a doubling of atmospheric CO₂ by

$$P_1 = \alpha P_0 \quad (3)$$

Changes in evapotranspiration will occur for two reasons, as a result of climatic change and through direct CO₂-induced changes in vegetation. If we use β to denote the fractional change in evapotranspiration

$$E_1 = \beta E_0 = \beta_1 \beta_2 \beta_3 E_0 \quad (4)$$

then β can be considered conceptually as the product of three factors, β_1 the change due to a change of climate, β_2 the change

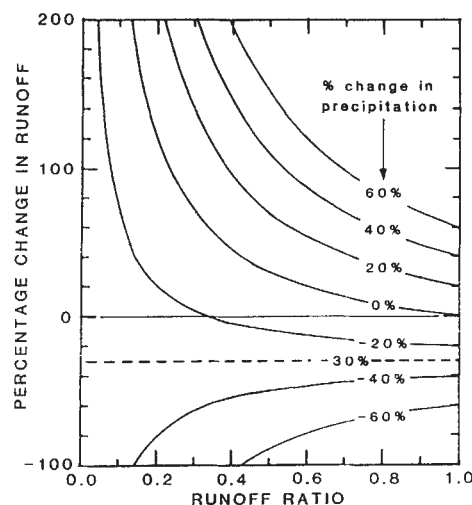


Fig. 1 Runoff changes due to changes in precipitation from a 60% reduction ($\alpha = 0.4$) to a 60% increase ($\alpha = 1.6$) assuming a maximum direct CO₂ effect on evapotranspiration ($a = 1$ in equation (5)) and no other changes in evapotranspiration ($\beta_1 = \beta_2 = 1$).

due to a change in the area of vegetated cover (due to either climatic change and/or direct effects of CO₂ on plant growth), and β_3 the direct CO₂ effect on evapotranspiration. For a doubling of CO₂, β_3 is given approximately (and conservatively) by

$$\beta_3 \approx 1 - 0.3a \quad (5)$$

where a is the fractional vegetated area of the drainage basin (see ref. 7). Manipulation of the above equations yields

$$\frac{R_1}{R_0} = \frac{\alpha - (1 - \gamma_0)\beta}{\gamma_0} \quad (6)$$

This result can be used to assess the relative sensitivity of runoff changes to changes in precipitation (α) and/or changes in evapotranspiration (β). In particular, direct CO₂ effects can be studied using equation (5). The relative sensitivity is given by

$$\left| \frac{\partial(R_1/R_0)}{\partial\alpha} \right| / \left| \frac{\partial(R_1/R_0)}{\partial\beta} \right| = \frac{1}{1 - \gamma_0}$$

Thus, runoff is always more sensitive to precipitation changes than to evapotranspiration changes, particularly for higher values of γ_0 . Individual sensitivities, however, are higher for small γ_0 . $\partial(R_1/R_0)/\partial\alpha$ always exceeds one, so that precipitation changes have an amplified effect on runoff, particularly in arid regions where γ_0 is small. This is a well-known result (see, for example, ref. 8). $|\partial(R_1/R_0)/\partial\beta|$ exceeds one for $\gamma_0 < 0.5$, so evapotranspiration changes only have an amplified effect for small γ_0 .

Figure 1 shows the change in runoff for various changes in annual mean precipitation in the presence of a maximum direct

Table 1 Runoff ratios for some of the world's major rivers, listed approximately in order of mean annual discharge

River	Runoff ratio	River	Runoff ratio	River	Runoff ratio
Amazon	0.47	Parana	0.20	Niger	0.19
Congo	0.25	St Lawrence	0.33	Indus	0.40
Yangtze	0.50	Irrawaddy	0.60	Yukon	0.48
Brahmaputra	0.65	Ob	0.24	Nile	0.10
Ganges	0.42	Amur	0.32	São Francisco	0.15
Yenisey	0.42	McKenzie	0.40	Rhone	0.46
Mississippi	0.21	Columbia	0.51	Vistula	0.22
Orinoco	0.46	Zambezi	0.12	Volga	0.28
Lena	0.46	Danube	0.30	Murray	0.04

The values presented (based on ref. 15) can only be taken as a general guide. Data from different sources can vary widely because of difficulties in estimating natural flows and catchment area precipitation.

CO₂ effect ($a = 1$). For smaller runoff ratios (that is, more arid regions) any given change in precipitation has an increasingly large effect on runoff. When $\alpha = 0.7$ and $a = 1$, equation (6) reduces to $R_1 = 0.7 R_0$, so that the runoff change is independent of the runoff ratio. For $0.7 < \alpha < 1.0$, runoff may increase or decrease depending on the value of γ_0 . For $\alpha > 1.0$ runoff always increases (as the precipitation and evapotranspiration changes

act in the same direction). The magnitude of the increase can be very large for small runoff ratios.

Table 1 lists runoff ratios for some major rivers. Values range from 0.1 or less for rivers which flow through arid regions up to more than 0.6 for some tropical rivers. In temperate latitudes, runoff ratios around 0.4 are fairly typical. Equation (6) may not be directly applicable to rivers with the lowest runoff ratios in Table 1. Some of these obtain most of their water from relatively small, high precipitation headwater catchments and then flow through arid and largely unvegetated regions. The runoff ratios given in Table 1 are for the whole drainage basin and may, in these cases, fail to represent conditions in the hydrologically-active parts of the basin.

Figure 2 shows the sensitivity of the runoff-change-precipitation-change relationship to the direct effects of CO₂ for various values of the runoff ratio. The magnitude of the direct effect can be varied by changing the parameter a . When $a = 0$ the direct effect is zero, and when $a = 1$ the direct effect is a maximum. The effects of climate related changes in evapotranspiration can also be studied using Fig. 2 since the range $a = 0$ to 1 corresponds to a range of β values from 1.0 to 0.7, that is to a range of reductions in evapotranspiration from 0 to 30%. The slopes of the lines in Fig. 2 are greatest for smallest γ_0 , showing that precipitation changes have maximum effect for small γ_0 . Thus, for small γ_0 (as noted earlier), precipitation changes may show a considerably amplified effect in runoff.

As an example, for a catchment with present runoff ratio of 0.2, the effect of a 10% reduction in precipitation with no direct CO₂ effect, to a 70% increase in runoff with a maximum direct CO₂ effect. For higher runoff ratios the range of possible runoff changes is less (see Fig. 2). In both Aston's⁶ study and the Idso and Brazel⁷ study, runoff ratios were low, around 0.16 in the latter case. This low value explains why Idso and Brazel obtained such vastly different results from Revelle and Waggoner³ when they incorporated the direct effects of CO₂. Table 1 shows that such low runoff ratios are unusual. Nevertheless, the direct CO₂ effect can still be substantial for more common runoff ratios. Idso and Brazel's case may also be unrepresentative of global conditions because they assumed precipitation reductions due to increasing CO₂ (following Revelle and Waggoner³). Climate model results all point to an increase in the intensity of the hydrological cycle and an overall (albeit slight) increase in global mean precipitation. There will, of course, be regionally and seasonally specific exceptions to this overall trend, but, in general, the effects of precipitation change and vegetation changes can be expected to reinforce each other. In some regions, therefore, we might expect very large increases in annual mean runoff, unless these are compensated by large climate-related increases in evapotranspiration.

The extent of this compensation is difficult to estimate because the magnitudes of climate-related changes in evapotranspiration due to increasing CO₂ are unknown. Over the land, evapotranspiration changes will result from several competing factors: warmer temperatures, changes in the length of the growing (evaporative) season, changes in wind speed, changes in cloudiness, and so on. Some of these factors may cause either increases or decreases in evapotranspiration. Although existing GCMs have recognized deficiencies in the way they model the hydrological cycle⁹, they still provide the best available guide to the climatic effects of increasing CO₂. All GCMs show an increase in the intensity of the global hydrological cycle. The increases vary considerably from model to model and are larger for models which show greater global-mean warming. For a doubling of CO₂ the global-mean increases in precipitation and evaporation range between about 3% (ref. 10) and 11% (ref. 4). These increases in evaporation are much less than the maximum direct CO₂ transpiration effect. However, there may be appreciable regional and/or seasonal departures from the global mean (see ref. 4) so, at least in some regions, we might expect the climate effects to modify the direct effects significantly.

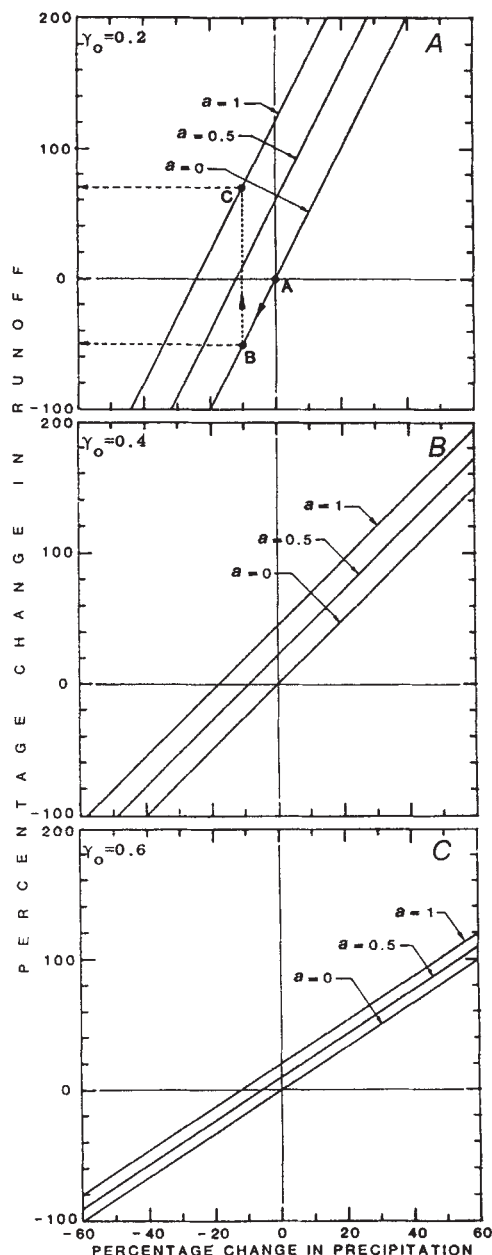


Fig. 2 Runoff changes due to precipitation changes for different evapotranspiration changes. $a = 0$ corresponds to no change in evapotranspiration, whereas $a = 1$ corresponds to a 30% reduction in evapotranspiration (the maximum direct CO₂ effect). The sensitivity to both precipitation change and evapotranspiration change increases for decreasing runoff ratio, γ_0 . Three values of γ_0 are shown (A, $\gamma_0 = 0.2$; B, $\gamma_0 = 0.4$; C, $\gamma_0 = 0.6$) spanning the most common range of observed values. A ($\gamma_0 = 0.2$) includes a specific illustration. The effects of precipitation change alone are determined by the $a = 0$ lines. The line AB, therefore, shows the effect of a 10% reduction in precipitation. The effects of evapotranspiration change are determined by paths parallel to the ordinate. Thus, BC corresponds to a range of evapotranspiration reduction from 0% (point B) to 30% (point C). Point C (and the lines $a = 1$ in general) corresponds to the maximum direct CO₂ effect, but the precise cause of the evapotranspiration change is unimportant.

The above analysis ignores several important factors. For example, the seasonal distribution of precipitation may be very different from that of evapotranspiration, and direct CO₂ effects may be diminished if the major contribution to runoff is in the winter months¹¹. Seasonal effects are, however, complex. For example, if E is defined as the residual of $P - R$ (equation (1)), then our results are independent of the seasonal distribution of precipitation. Direct CO₂ effects may also be reduced if increasing CO₂ causes an increase in leaf temperatures, in individual plant leaf areas or in the total vegetated area of a catchment^{11,12}. More complex catchment models (such as the statistical models of Wright¹³ and Jones¹⁴, or deterministic models like that used by Aston⁶) are required to study these effects. Our study does, however, provide a basis for first estimates of annual mean runoff changes for any river whose runoff ratio is known, and allows one to identify regions most likely to experience large runoff changes due to increasing CO₂.

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Gypsum precipitation from cold brines in an anoxic basin in the eastern Mediterranean

Scientific staff of Cruise *Bannock* 1984-12*

In 1968 hot brines from which gypsum had precipitated were discovered in the Red Sea, in a divergent plate boundary setting with hydrothermal activity¹. We now report the discovery of cold brines in an entirely different geodynamic situation, a convergent plate boundary in the eastern Mediterranean, where the heatflow is very low² and no hydrothermal activity occurs. Decimetric pure gypsum crystals precipitate from the cold brines, which occupy the bottom of a rimmed anoxic basin near the southern edge of the Mediterranean Ridge, close to the Sirte Abyssal Plain. Nucleation of the crystals does not occur at the surface, but from the deep brines, because the salinity of the water is normal down to the depth of -3,000 m. We propose the name 'Bacino Bannock' for this basin, after the Consiglio Nazionale delle Ricerche R/V *Bannock* from which the bottom-water brines were surveyed and sampled.

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Another anoxic basin containing highly saline bottom waters was discovered last year in the western Strabo Trench, north of the Mediterranean Ridge, and named Tyro Basin³. Bacino Bannock differs from Tyro in that gypsum precipitation is only recorded in the former.

Bacino Bannock was discovered using Seabeam and Seamarc I during the *Robert D. Conrad* Cruise 25-06 (April 1984). In September 1984, *Bannock* Cruise 84-12 completed a narrow-beam echosounder bathymetric survey of the basin, obtained 10 cores and two dredges, and conducted three CTD (conductivity/temperature/depth) stations and one Nansen-bottle hydrocast. Navigation on both *Conrad* and *Bannock* was by Loran-C and transit satellites.

Bacino Bannock is an irregular depression (Fig. 1) about 8 nautical miles (15 km) in diameter, within the irregular 'cobblestone topography' of the Mediterranean Ridge. A prominent bulge occupies the centre of the basin, surrounded by a moat-like depression. The Bannock sampling programme was concentrated on, and to the west of, the central bulge, where the depression is deepest.

Sediment cores (Fig. 2) from 3,000 m and shallower contain the typical eastern Mediterranean pelagic stratigraphy of sapropels and tephra. However, bottom sediments collected deeper than 3,200 m are black to dark grey and smell strongly of hydrogen sulphide. This anoxic environment contains a stratigraphy of tephra layers, breccias, carbonate sands and graded sands and silts. All samples examined at sea were richly fossiliferous, with varying proportions of planktonic and displaced benthic foraminifera (*Asterigerinata mamilla*, *A. planorbis*, *Asterigerina* sp., *Gavelinopsis lobatulus*, *Rosalina globularis*, etc.), calcareous nannoplankton and siliceous plankton. Anoxic sediments are over 9 m (implying a sedimentation rate of 10 cm kyr⁻¹ in our longest core) and range in age from Holocene, probably to middle Pleistocene, as based on a combination of calcareous nannofossil biostratigraphy (*Gephyrocapsa oceanica* Zone) and planktonic foraminiferal palaeoecology.

A basal normal contact between anoxic and pre-anoxic sediments was not recovered. However, two cores (below 3,150 m, 05 and 06 GC) recovered oxic pelagic sediments of middle and late Pliocene age unconformably beneath Holocene anoxic sediments and in a breccia, respectively.

Abundant gypsum in cores and dredge hauls was the most surprising discovery at Bacino Bannock. In cores, individual crystals range in size from microscopic to the full diameter of the core barrel (7 cm). The dredges recovered enormous masses of interlocked gypsum crystals, up to 0.5 m across (Fig. 3). Both dredges were caught up in the gypsum and required more than 5 tons of pull and several hours (more than 24 h at station 11 D) to be freed. These composite gypsum masses were only recovered from the steep western wall of the central bulge where they adhered to a hard substrate of grey Pleistocene marlstone. Individual crystals in cores and dredges were both tabular and bipyramidal and many contained inclusions concentrated along crystal planes. Inclusions consisted of pelagic debris such as planktonic foraminifera (Fig. 4), including *Globigerina bulloides*, *Globigerinoides ruber* and *Orbulina universa*, pteropods, including the Holocene species *Cavolinia gibbosa*, and *Cavolinia inflexa*, *Clio pyramidata* and *Atlanta* sp., and mineral grains.

Rubbery mats, similar to those described from the Tyro Basin³ and consisting of organic fibres containing diatoms, planktonic foraminifera and mineral grains, were found in both cores and dredges. In cores, the mats were recovered as horizontal disks, presumably cut from larger mats as though by a biscuit cutter; in dredges they occurred between crystals, with no preferred orientation.

CTD measurements indicate normal Mediterranean deep-water down to 3,000 m (the length of the conducting hydro-cable on board). A 15-kHz echosounder recorded horizontal reflectors within the water column at depth of 3,228, 3,428 and 3,325 m, suggesting locally-high gradients in temperature, salinity and/or particulate matter. During coring and hydrocast operations, the depth of the instrument was monitored by an acoustic pinger

Fig. 1 Bathymetry of Bacino Bannock as determined by 3.5-kHz narrow-beam echo sounder and Seabeam. Contour interval is 100 uncorrected metres. Sampling stations are: D, dredge; GC, gravity core; PC, piston core; WB, water bottle cast. The brine (light stipple pattern) is confined beneath the 3,150-m contour. Navigation is by Loran-C, adjusted to satellite coordinates. Note location of Tyro and Kretheus Basins, where anoxic bottom conditions were discovered last year³ and of Katia Basin, where such conditions were found during the present investigation.

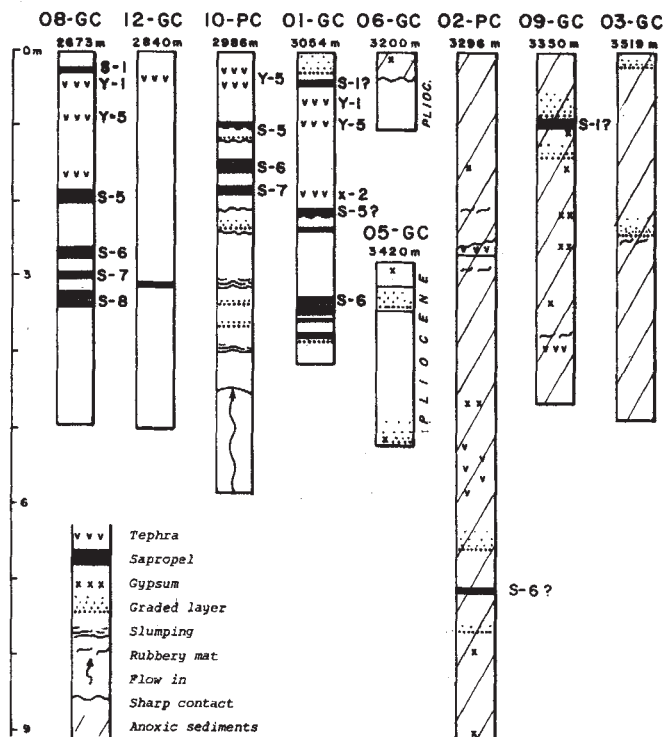
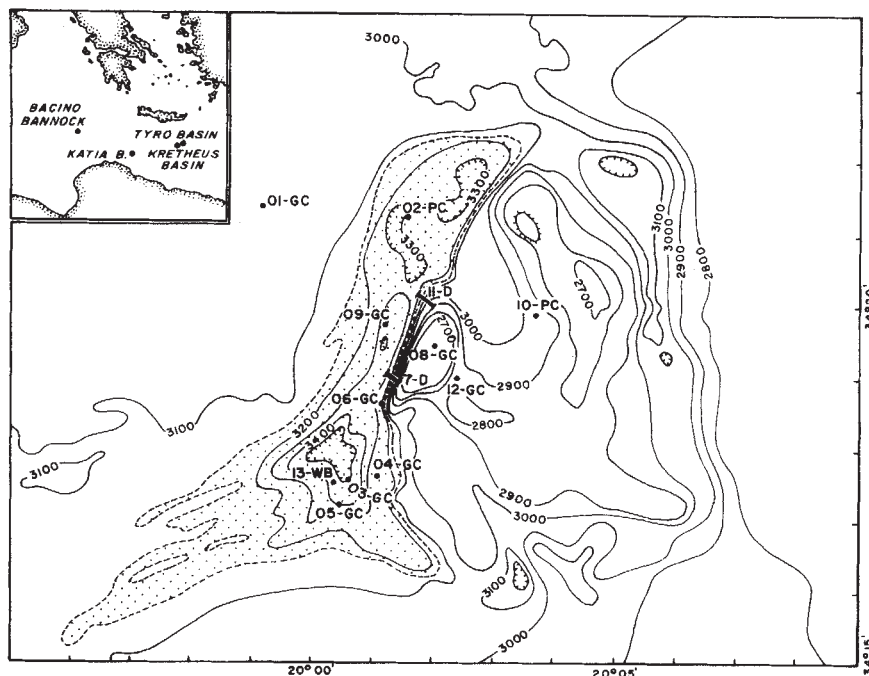


Fig. 2 Simplified core logs from Bacino Bannock. Cores shallower than 3,000 m were recovered from a normal eastern Mediterranean pelagic sequence including sapropels and tephra, whereas cores deeper than 3,200 m were recovered from grey and black sediments.

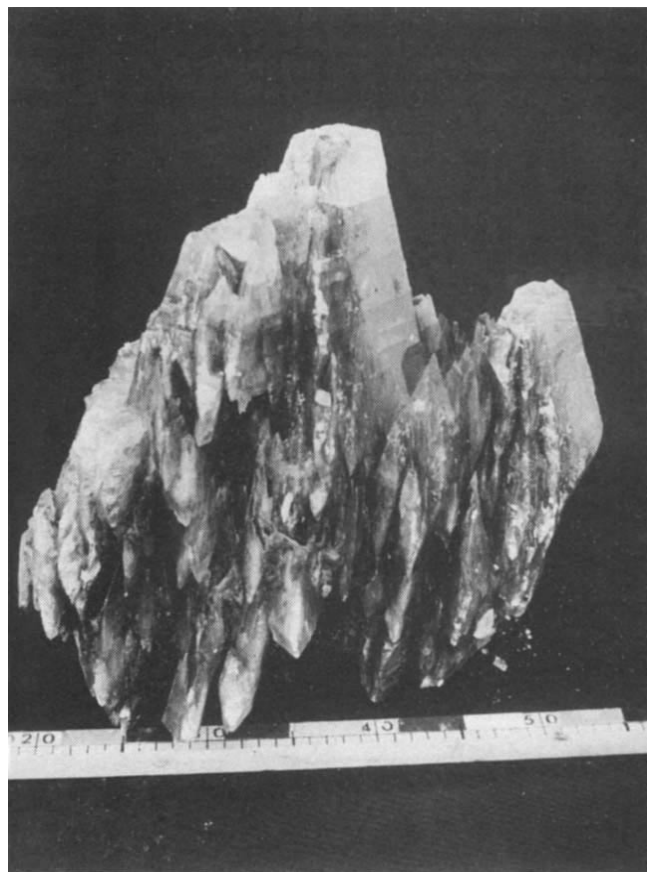


Fig. 3 Photograph showing a large mass of interlocked gypsum euhedra recovered in dredge 7-D (scale in cm). The size and perfection of the euhedra suggest that this gypsum was recently crystallized from a hypersaline brine rather than being relict from the Messinian.

displayed on a facsimile recorder as a depth versus time plot. As the pinger neared the bottom of the basin, a break in the slope appeared on the plot, which we believe resulted from an abrupt change in sound velocity occurring somewhere between 3,100 and 3,340 m.

We infer from sound velocity contrast measurements that the black muds, stinking of hydrogen sulphide, are best explained by the presence of anoxic hypersaline bottom water as has been recovered from the Tyro Basin^{3,4}. The upper level of the hypersaline brine is inferred to be at the level of the shallowest closed contour, 3,150 m. This is in agreement with echosounder mid-water reflectors, sound velocity contrast and water depths from which anoxic and oxygenated sediments were recovered. The

estimated depth of the interface is consistent with CTD observations that showed no increase in salinity down to 3,000 m. The bottom-water layer occupies an area of 36 km² and has a volume of ~4 km³.

The most probable source of hypersaline bottom water is by leaching from latest Miocene (Messinian, approximately 6–5 Myr BP) evaporites. Although we did not sample strata of this

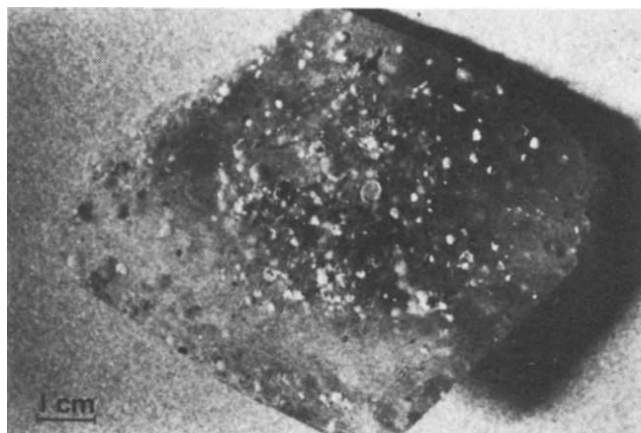


Fig. 4 Photograph showing a euhedral gypsum crystal recovered in core 09 GC, Sec. 1, 80 cm from core top. The numerous biogenic inclusions consist of planktonic foraminifera.

age, several pieces of evidence suggest that Messinian sediments must be at, or close to, the sea floor. First, a single channel reflection profile 10 km west of Bacino Bannock shows a regional reflector (layer M) that correlates with the top of Messinian evaporites^{5,6} at sub-bottom depth of 100 m. Second, sedimentation rates calculated for the plateau cores (01, 08 and 10) range from 1.5 to 2 cm kyr⁻¹. Extrapolating this sedimentation rate to 5 Myr BP gives a maximum thickness of 100 m for the Plio-Quaternary section.

At present, gypsum appears to be precipitating from the hypersaline brine at the seafloor. This is indicated by the size and perfection of the crystals, as well as the age of their substrates and inclusions. Gypsum found down-core could have precipitated either from bottom water, before burial at the sediment-water interface, or from pore water, after burial within the sediment.

The degree of anoxia and/or hypersalinity may have varied with time as shown by the change in colour intensity down-core, and by the irregular distribution of gypsum crystals. Some of the sediments are indeed re-sediments, as suggested by the occurrence of millimetric silty laminae, high sedimentation rate and the presence of benthic foraminifera, mostly displaced down-slope. It is possible that the variations in the degree of anoxia were responses to eastern mediterranean-wide hydrographic conditions, as well as to local phenomena. A jet-black layer at 711 cm in core 02 PC, which may correlate with sapropel S-6, contains a cold-water fauna dominated by *G. bulloides* and *Globoquadrina dutertrei*. This fauna characterizes the middle Pleistocene 'glacial anoxia' event observed throughout the eastern Mediterranean⁷. The base of this core is referable to the middle Pleistocene *G. oceanica* nannofossil zone.

The Mediterranean Ridge is marked by numerous subcircular depressions, some of which have been interpreted as resulting from dissolution and collapse of Messinian evaporites^{8,9}. Thus, it is reasonable to expect strong density stratification and consequently, anoxic bottom waters in other basins. Indeed, Jongsma *et al.*¹³ have described anoxic conditions present now in the Tyro Basin, and up to a few thousands years ago in the adjacent Kretheus Basin, both in the Strabo Trench. On Bannock cruise 84-12, we also identified anoxic conditions (cores 29 PC and 30 GC) that lasted until a few thousand years ago in basins of the Katia area near the Libyan continental margin.

In conclusion, we note that the unique aspect of Bacino Bannock is the deep water precipitation of the gypsum under very specific geological, chemical, hydrographical and physiographical conditions.

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Abundance variation of iridium and trace elements at the Permian/Triassic boundary at Shangsi in China

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Despite recent searches for anomalous iridium(Ir) concentrations at the Permian/Triassic boundary (P/Tr) of marine sediments¹⁻³, no Ir above a level of 0.5 p.p.b. (parts per 10⁹) has been found. Detailed event-stratigraphical research has revealed a comparatively high platinum-group enrichment and geochemical anomalies of trace elements in the P/Tr boundary clay at Meishan, Changxing County, Zhejiang Province, China⁴. We report here an investigation of Ir and trace-element abundances in the P/Tr boundary layer of Shangsi section, Guangyuan County, Sichuan Province (Fig. 1). An Ir value of 2 p.p.b. detected in the boundary layer supports the occurrence of an extraterrestrial event at the end of the Permian.

The Shangsi P/Tr layer is located in a road section ~1 km from the town of Shangsi. This section has been extensively investigated, so that stratigraphical sequences of the Permian and Triassic are clear. However, opinions differ as to the demarcation of the P/Tr boundary; most hold that the boundary is close to the horizon where sample AG253 was collected⁵.

We concentrated on the P/Tr boundary sediments of total thickness 4 m and most of the samples were collected from shales and clay. The stratigraphical sequence of measured section is shown in Fig. 2.

Note that in South China, *Pleurodoceras* sp., *Pseudotiroilites asiaticus* and *Neogondolella changxingensis* in layer 3-5 are index fossils usually present at the top of the Changxingian. However, a new species of *Claraia* was also found. Hence, the different fauna groups provide several options on where to put the P/Tr boundary in the Shangsi section. For instance, the base of the Triassic has been placed between layer 2 and 3, according

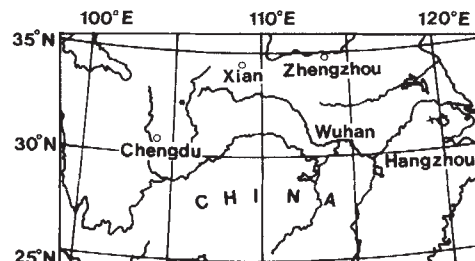
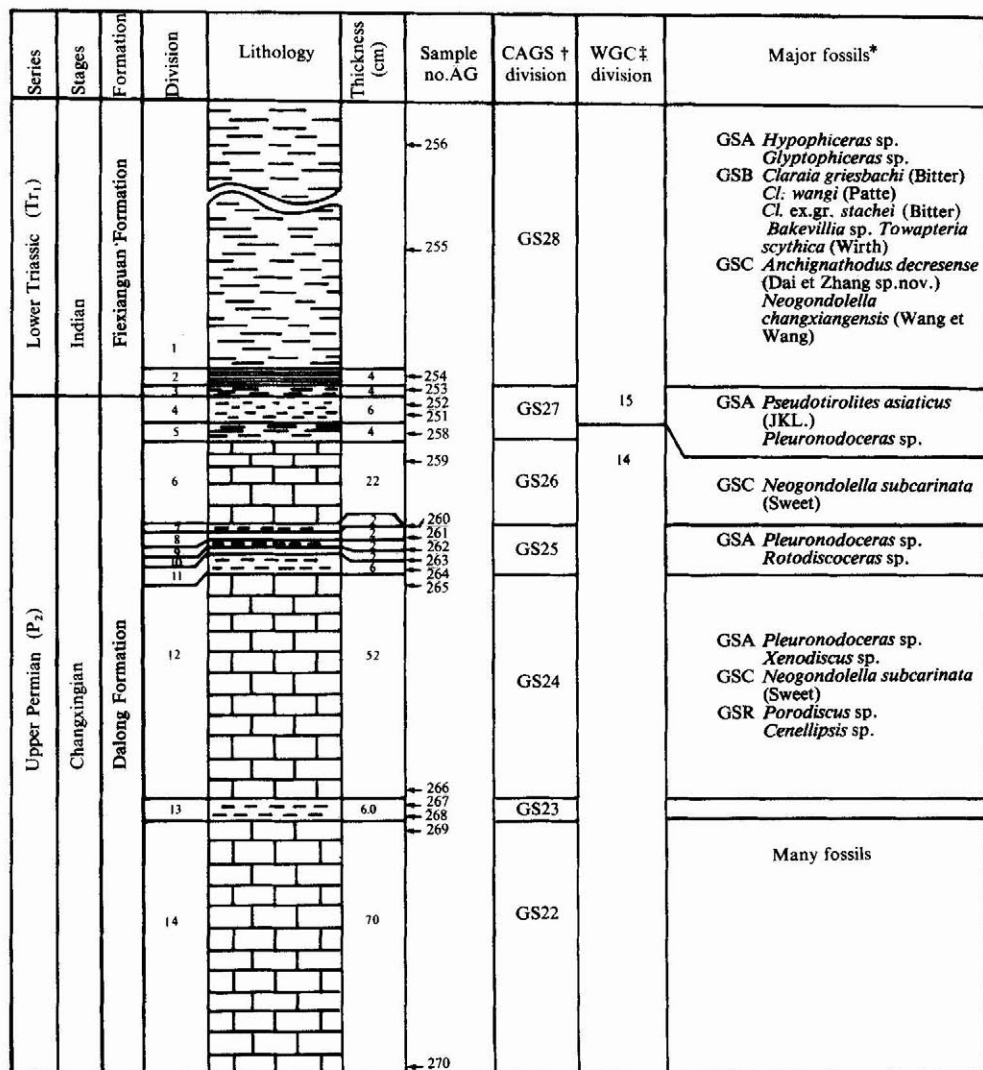


Fig. 1 Sketch map of the locations of two Permian/Triassic sections. *, Shangsi section, Guangyuan; x, Meishan section, Changxing.

Fig. 2 P/Tr boundary stratigraphical section at Shangsi, Guangyuan. *, From Li Zishun; †, Chinese Academy of Geological Science; ‡, Wuhan College of Geology. Divisions: 1, silty shale; 2, grey shale; 3, grey-black calcareous shale; 4, light coloured shale; 5, grey-black calcareous shale; 6, grey siliceous limestone; 7, grey shale; 8, dark-grey calcareous shale; 9, grey shale; 10, dark-grey siliceous shale; 11, grey shale; 12, dark grey siliceous limestone; 13, grey shale; 14, dark-grey siliceous limestone.



1 : 10

to the ammonites. But, from the bivalves, the P/Tr boundary is lower, whereas conodont assemblage favours moving the P/Tr boundary much higher.

We analysed 10 samples from Shangsi P/Tr section by instrument neutron activation analysis (INAA); the analytical results are shown in Table 1. To check the reliability of INAA, some samples of Cretaceous/Tertiary boundary clay from several sections in Western Europe were also measured. The results seem to concur.

From the 10 analysed samples, only one (AG253) shows an Ir concentration of 2.0 ± 0.5 p.p.b., which is based on two laboratory runs, the lower limit of detectable Ir in the analyses is about 0.5–0.6 p.p.b. Note, that the Shangsi samples, investigated by Zhang *et al.*³, were taken from layers GS25 and GS24 (equivalent to layers 11 and 12 in the present study), which lies ~0.5 m lower than sample AG253.

Table 1 shows the data sets of the abundance variation of 14 other elements. There are two variation trends: elements of the

Table 1 INAA results of samples at Shangsi section

Element	AG256	AG254	AG253	AG252	AG251	AG258	AG260	AG262	AG264	AG268
Ir	ND	ND	2.48	ND	ND	ND	ND	ND	ND	ND
Ni	42.1		63.2			43.8		46.9	38.3	35.9
Co	15.2	25.8	17.5	2.43	1.83	21.6	5.99	7.25	4.13	1.52
Fe	3.25	2.43	3.62	1.63	1.57	2.29	2.40	2.21	1.96	1.75
Cr	64.1	71.9	88.7	7.85	3.80	57.1	4.98	6.79	2.18	3.50
Sb	0.81	0.91	1.2	0.57	0.47	1.18	0.36	0.38	0.45	0.18
U	2.31	4.08	5.56	9.46	9.11	4.49	4.68	6.95	11.9	13.3
Th	11.1	12.4	13.6	39.5	42.2	13.8	38.8	47.2	49.5	54.5
Rb	136	73.9	162	126	114	107	119	116	119	115
Cs	7.70	4.66	9.64	13.4	11.0	6.09	17.9	12.0	11.4	12.0
Sr	104	1010	160	540	574	875		158	168	113
Ba	294	180	314	70	55.5	235	159	71.1	111	141
Zr	270	198	349	476	610	270	326	936	1020	693
Hf	3.68	3.49	3.71	9.69	10.1	4.20	7.35	17.3	16.7	12.7
Ta	0.91	0.69	0.96	1.68	1.67	0.87	2.11	2.22	2.58	3.39

ND, Not detected; all measurements are p.p.m. except Ir (p.p.b.) and Fe (%).

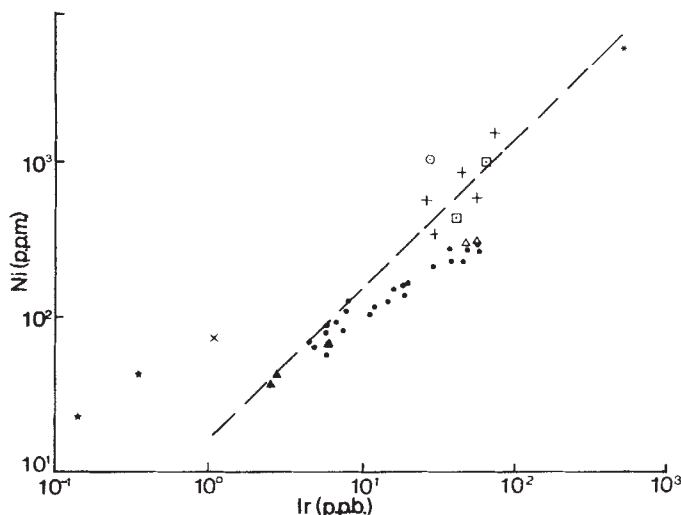


Fig. 3 Relationship between Ni and Ir content in the boundary rocks of the Permian/Triassic (P/Tr); Cretaceous/Tertiary (K/T). X, Crust; *, CI; †, Tunguska metallic spheres⁶; ▲, P/Tr boundary beds at Shangsi; ★, average Danish K2 E1 rocks (from ref. 8); ●, average Danish K/T boundary bed (from ref. 9); ○, average Danish K/T boundary bed (from ref. 8); □, average Danish K/T boundary bed (from ref. 7); △, average Danish K/T boundary bed (from ref. 10).

first trend reach maximum or sub-maximum values at AG253. This is the situation for siderophile elements (Ni, Cr, Fe), chalcophile (meteorite) elements (Sb) and a few lithophile elements (Ba, Rb). Most of the lithophile elements (Cs, Rb, Hf, Ta, Zr, Th, U and so on) belong to the second variation trend, which shows decreasing elemental concentrations in sediments of younger geological ages. So, there is probably little or no correlation between the Ir anomaly and the variation trend of most lithophile elements. However, the variation of Fe, Ni, Cr and so on shows a close correlation to the Ir anomaly in sample AG253.

Ganapathy⁶ has used the Ni/Ir ratio to distinguish metallic microspherules of an extraterrestrial origin from those of terrestrial origin: on an Ni-Ir diagram, microspherules of extraterrestrial origin show regular linear distribution. Figure 3 shows the Ni and Ir contents of CI carbonaceous chondrites, Tunguska microspherules and Danish Cretaceous/Tertiary boundary clay. These points tend to an approximately linear distribution. The slope of the dashed line in Fig. 3 reflects the cosmic ratio of Ni and Ir. The Ni/Ir points of Shangsi and Meishan samples plotted on Fig. 3 fall into a linear distribution area. Figure 3 also shows that the Ni and Ir values of the crust, Upper Cretaceous beds and Lower Palaeocene beds from Denmark deviate from the linear distribution.

The evidence from the mass extinction of biocommunities, the Ir anomaly of AG253, the variation in the abundance of trace elements, and the comparison between the platinum anomalies at P/Tr boundary at Meishan, Changxing⁴ and those at the end of Cretaceous throughout the world, supports the conclusion that the 2 p.p.b. Ir anomaly in AG253 reflects the effects of an extraterrestrial event that occurred at the end of Permian.

Study of the Ir anomaly may provide a new approach to stratigraphical subdivision and correlation. The above arguments place the P/Tr boundary at the Shangsi section at the base of layer 3.

This paper is only a preliminary report and more detailed studies are being planned.

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Timing of deglaciation from an oxygen isotope curve for Atlantic deep-sea sediments

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The exact timing of deglaciation is an important issue in the context of climatic feedback involving the deglacial rise in atmospheric CO₂ seen in ice cores¹⁻⁴. We have produced evidence, from box cores taken in the equatorial Atlantic, that the last deglaciation occurred in two major steps which were separated by a brief pause⁵. Here we propose that this pause is equivalent to the period of glacial readvance known as 'Younger Dryas', which lasted from about 11 to 10 kyr BP. If our correlation is correct, then the initiation of deglaciation occurred after 14 kyr BP, which disagrees with most other deep-sea timescales.

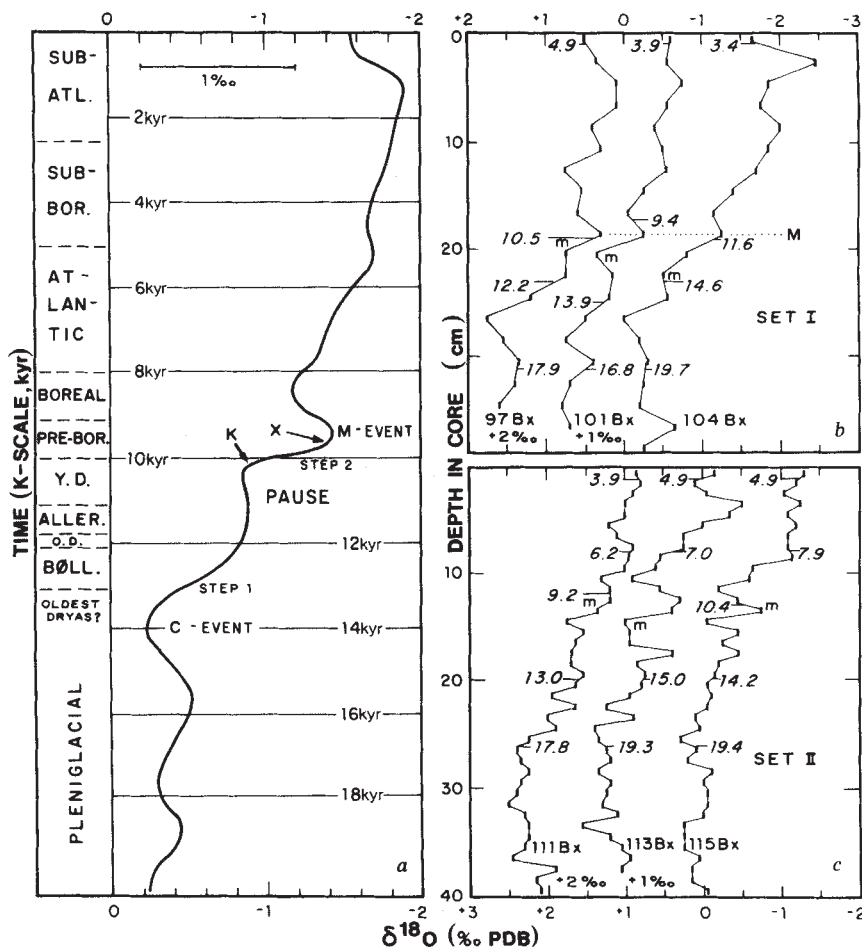
Two-step deglaciation has been proposed previously, notably by Duplessy *et al.*⁶ who based their conclusions both on extensive micropalaeontological work at the Institut de Géologie du Bassin d'Aquitaine, and on new radiocarbon-dated δ¹⁸O signals measured at Gif-sur-Yvette. Berger⁷ proposed that the Atlantic deep-sea deglaciation record is correlated, as a two-pulse feature, with the record in the Gulf of Mexico⁸ which clearly shows a double-pulse meltwater input⁹. Independently, Ruddiman and McIntyre¹⁰ came to the conclusion that deglaciation occurred in major pulses, based on the micropalaeontological record in North Atlantic sediments.

The pulsed nature of the last deglaciation, then, appears to be well established. However, strong disagreement persists concerning the matter of timing^{11,12}. Several papers⁸⁻¹⁰ date the beginning of deglaciation well before 15 kyr BP. In contrast, our data from the Pacific suggested that deglaciation commenced after this time¹³. Also, the data given for the Gulf of Mexico by Emiliani *et al.*¹⁴ show no major influx there before 14 kyr BP. The timescale of Duplessy *et al.*⁶ has deglaciation beginning near 15,000 BP. Sarnthein *et al.*¹⁵ suggest that "the Termination I continued from about 16,000 to 8,500 yr BP", but consider that tropical planktonic foraminifera "indicate a delayed ocean warming which did not start till 14 kyr BP", in the Northern Hemisphere.

The chief problem with accepting any of the published timescales is the varying quality of radiocarbon ages in deep-sea sediments; despite special precautions, problems persist. The well-dated core of Duplessy *et al.*⁶ (Core CH 73139C, west of Ireland), for example, shows virtually identical radiocarbon ages for the section between 110 cm and 145 cm, namely, 11.5-11.7 kyr, as well as a major break in sedimentation rate near 200 cm. These results suggest that the record suffers from redeposition events and/or other disturbances. Although the ages down to Ash Layer 1 appear correct (the date of this layer is near 10 kyr), the older ages downcore are suspect. Within the crucial interval centred on 15 kyr, little or no planktonic carbonate may have been delivered to this site because of its northern position¹⁰.

Our box-core data (Fig. 1) allow an independent assessment of the reliability of bulk carbonate ages. For this purpose, the cores are correlated on the basis of oxygen isotopes, with support

Fig. 1 Timescale of deglaciation based on stacked $\delta^{18}\text{O}$ record of three equatorial cores from the Atlantic (a). K, the fixed point, taken as end of Younger Dryas, at 10.2 kyr. X, The level where radiocarbon ages of bulk carbonate are 11 kyr on average. The K-timescale is based on position of point K and linear sedimentation rate. b, $\delta^{18}\text{O}$ records from three box cores from the equatorial Atlantic, and ^{14}C dates in kyr. m, Appearance of *G. menardii*. M-event, a 'warm' excursion in the $\delta^{18}\text{O}$ record at the end of deglaciation thought to be correlative with the Gulf of Mexico meltwater event of refs 8, 9, 14. c, Similar to b but cores from tropical South Atlantic. $\delta^{18}\text{O}$ measures against PDB standard (*G. ruber*, pink).



from the appearance of the planktonic foraminifera *Globorotalia menardii*, a Holocene marker species. The data plotted in Fig. 1b,c, demonstrate that there are two sets of cores whose sedimentation rates are almost identical. Within each of these sets, correlation coefficients are very high (≥ 0.9). They are diminished when differences in overall sedimentation rates are assumed, as we established by systematic numerical experiments. Depth levels within these sets, then, are more or less synchronous. The synchronicity, presumably, tends to deteriorate with depth-in-core, as small differences in sedimentation rates lead to increased divergence in age. If we accept synchronicity within sets for Holocene sediment (< 11 kyr), a typical standard deviation about the mean of radiocarbon ages is 0.5–0.9 kyr (Table 1, second and fourth column). This uncertainty seems to reflect the confidence which can be put in the dating of isotope signals based on bulk radiocarbon in these cores. The scatter is a result of sampling and counting errors, mixing processes, and redeposition of fine-grained carbonate. These radiocarbon age data, quite clearly, cannot be used for arguments involving time-resolution of $< 1,000$ yr.

In the following analysis, we use the radiocarbon dates for general guidance: the detailed timescale we propose is based on the correlation of climatic signals. We argue that the radiocarbon dates must be considered maximum ages. Our chief evidence is the high average age of surface sediments (4.1 and 4.6 kyr, Table 1). High surface ages have been reported previously from deep-sea carbonates, they were explained by benthic mixing processes^{16,17}. To a first approximation the age of the mixed layer represents the residence time of particles in the mixed layer reservoir, whose thickness should be close to $s \times t$, where s is the sedimentation rate and t the age. For our cores ($s = 1.5$ to 2 cm kyr^{-1}) this thickness would be between 7 and 8 cm. Such a high value conflicts with expectations for these areas¹⁸, especially for the set II region where ocean productivity is low and benthic stirring therefore reduced¹⁹. We suggest that the old surface ages result not from vertical benthic mixing so much

as from the lateral advection of old fine-grained carbonate, delivered from elevated areas. Direct evidence for 'old' fines is in the comparison of coarse fraction ages with bulk ages (Table 2). The bulk ages are older by about 2 kyr, on average. The fines, then, must be older than the coarse material by 2.5 to 3 kyr. Discrepancies in the ages of coarse and fine fractions of deep-sea carbonates have been reported previously^{20,21}. The point is that the climatic signals, carried in the isotope composition of planktonic foraminifera, is embedded within sediment that is too old, as far as the signal's timescale is concerned.

Table 1 Average ^{14}C ages (in kyr) at selected depth levels, in cores of set I and set II, and comparison with K-scale ages (based on climatic record)

Depth (cm)	Set I	K-age (Δ)	Set II
1	4.1 $\pm$ 0.8	0.5 (3.6)	4.6 $\pm$ 0.5
8			7.0 $\pm$ 0.9
19	10.9 $\pm$ 0.6	9.7 (1.2)	13.3 $\pm$ 1.1
25	14.4 $\pm$ 1.4	12.8 (1.6)	18.0 $\pm$ 0.9
31	18.1 $\pm$ 1.5	15.9 (2.2)	

K = 10.2 kyr; M = 9.5 kyr; Δ = difference between set I age and K age.

Table 2 Radiocarbon ages (in kyr) of bulk carbonate and coarse fraction in INMD 115 Bx

Depth (cm)	Bulk age	Coarse fraction	Fine fraction*
0–2	4.85 $\pm$ 0.17	2.24 $\pm$ 0.11	5.62
7–9	7.87 $\pm$ 0.12	6.90 $\pm$ 0.13	8.11
19–21	14.17 $\pm$ 0.19	11.4 $\pm$ 0.17	14.93

* Fine fraction calculated (based on % carbonate and % sand): carbonate, 90.7, 91.4, 89.5; sand, 20.7, 18.3, 19.4.

We now consider the land-based timescale of climatic change for deglaciation and the Holocene to date our isotope signal (Fig. 1a). The resulting timescale should be younger than the corresponding deep-sea radiocarbon ages, by an amount comparable to the difference between bulk- and coarse-fraction ages. Of course, the line-up of climatic changes on the land and in the sea should match closely.

Figure 1a is derived from stacking the signals of cores 97, 101 and 104 (Fig. 1b). The shape of the isotope signal is very similar to one published earlier, from the same region, based on Albatross cores²², but without age control. The radiocarbon measurements suggest an age near 11 kyr for step 2 (point X). According to our argument, this age represents a maximum for this climatic event. The most nearly synchronous and also the younger event on land, marking a time of rapid change from cold to warm conditions, is represented by the transition from the Younger Dryas cold period to the Preboreal warm interval. Thus the 'cold spot' preceding step 2 in our signal is fixed as the Younger Dryas, which is centred on 10.5 kyr. The entire timescale is now derived by assuming zero age for the surface, and linear sedimentation rate to the Younger Dryas fix-point. With this assumption, an excellent fit is obtained with the Norden timescale²³, based on Scandinavian climatic history (Fig. 1). The differences between the set I ages and the ages found by climate correlation are of the expected magnitude (except for the benthic mixed layer proper) (Table 1, third column).

Our new timescale puts the start of deglaciation just after 14 kyr BP (instead of 15 kyr, based on raw ¹⁴C data). Step 1 is centred on 13 kyr and step 2 on 10–9.5 kyr. Our deglaciation steps compare favourably with the micropalaeontological 'warming' steps of Ruddiman and McIntyre¹⁰, established for the northern Atlantic. The correlation of the deglacial pause with the Younger Dryas, a major cold event on land, was previously suggested by Duplessy *et al.*⁶. The end of the pause is at point "K", which is taken to be 10.2 kyr BP. This event fixes our timescale, by linear interpolation and extrapolation. Point K slightly predates the Ash Layer 1 of Ruddiman and McIntyre²⁴, dated at 9.8 kyr BP in ref. 6.

We refer to the timescale adopted in Fig. 1 as the 'K-scale'. Its veracity ultimately rests on a climatic correlation (hence K for Klima), namely, the fit of the pause between the two deglaciation steps to the Younger Dryas cold period. This correlation was first suggested to us by Johnson²⁵.

Major questions arising from the new deglaciation scale are: what is the positive feedback mechanism which was responsible for the steepness of the two steps; why did deglaciation pause halfway through its completion; and is the published information for ice retreat on land compatible with the deep-sea oxygen isotope record when dated in the manner shown? The evidence for pulsed meltwater input, combined with the short timescale advocated here, makes it likely that the sought-for feedback mechanisms involved intermittent slowing of vertical ocean mixing (the 'Worthington effect')²⁶. Such slowing would affect the carbon dioxide content of the atmosphere²⁷, and hence the direction and the rate of climatic change. There is one possible way the 'early-deglaciation' timescale^{10,15} could be resurrected: if it could be shown that lack of carbonate production (for example, owing to a meltwater lid) produced a carbonate hiatus in the crucial period, near 15–16 kyr, so that a planktonic isotope signal was not recorded, even in the equatorial Atlantic.

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A prehistoric calendrical site in Argyll?

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The hypothesis that many prehistoric standing stone sites in Britain were set up, in relation to natural horizon marks, as astronomical observing instruments has been controversial for the past two decades. Although based primarily on statistical arguments, whether or not it stands will depend on the outcome of practical tests; it must prove able to predict at some sites the occurrence of archaeological features which can then be found by excavation. We report here on the findings at Brainport Bay in Argyllshire of artificial features pointing towards the midsummer sunrise that have been dated suitably early and of another feature indicating alignment towards the sunset at the equinox, that was first predicted and then discovered. These results seem to provide strong support for Thom's hypothesis that calendrically useful solar markers existed in Scotland at least as early as the Bronze Age.

At Brainport Bay, near Minard on Loch Fyne, one of us (P.F.G.) discovered in 1976 a series of artificial stone structures which were first interpreted as a settlement site, but the absence of clear signs of dwellings or of midden material made an alternative hypothesis desirable. Since then, the possibility of their forming an alignment towards midsummer sunrise has been considered, which seemed to explain several hitherto inexplicable features^{1,2}. The presence of flint flakes and artefacts and of shattered quartz fragments on the various parts of the site confirmed Mesolithic or Neolithic activity, but there were also much later iron objects and slag. The supposed alignment consists of three main features running from north-east to south-west, namely the 'main outcrop' (with paving on it and two lower revetted 'terraces' at its south-west end), the 'observation boulders', 9 m further south-west and, 50 m further in the same direction, the 'back platform', a few metres higher up (Fig. 1).

From between the two boulders one has a striking view north-east up Loch Fyne, and a partly artificial rock notch at the south-west end of the main outcrop (1.38 m deep and 1.30 m wide at the top) frames the only two distant peaks to be seen—Beinn Oss and Beinn Dubhcaig, 28 miles away near Tyndrum. A small socket was found immediately in front of this notch and another up at the north-east end of the outcrop; two stone slabs 1.28 m and 1.46 m long, which fit these sockets well, were

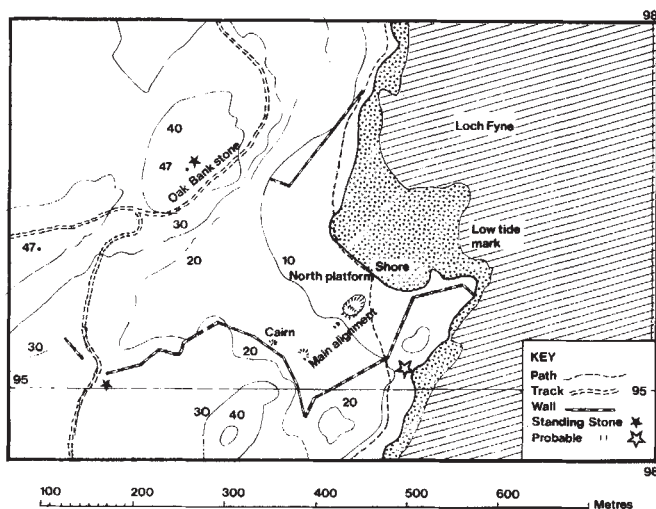


Fig. 1 General plan of Brainport Bay showing the main alignment (with the Back Platform at the south-west) and some nearby outlying features. The contours are in metres above mean sea level.

found on the buried land surface nearby. All these features—boulders, notch and sockets—lie on a straight line pointing to the mountain peaks behind which the midsummer Sun rises (see Fig. 3.5 in ref. 2). The arrangement looks like the sights of a huge rifle aimed at the solstitial Sun, but to be of practical as opposed to ceremonial use, the cleft between the two peaks would have to have been used. Measurements by Thom showed that this cleft has a declination of $+23^{\circ}22'$, slightly before the solstice of 1800 BC; then it would have marked the upper limb of the Sun rising on two occasions about 32 days apart, before and after midsummer (see p. 134 and Fig. 3.8 in ref. 2).

The third element in the alignment, the back platform, consists of a rock outcrop on the northeastern edge of higher ground and that has been modified with revetting and paving; there is a broad flat area behind it. From here there is a dramatic, though generalized, view of the midsummer dawn, but not through the rock notch, which is now too low.

Excavations in 1982 revealed a buried sandy soil behind the back platform on top of which were struck flint flakes; a ^{14}C date of 1060 ± 80 BC (GU 1705) was obtained for charcoal associated with the flints, equivalent to the fourteenth century BC in calendar years³. The revetted edge of the larger terrace next to the main outcrop was found to lie on the same buried soil (Fig. 2). Thus, the artificial elements of the main alignment have been fairly securely dated to the mid-second millennium BC or earlier. Excavations at the 'observation boulders', however, showed that, contrary to previous belief (see p. 134 and plate 3.6 in ref. 2), these have been in position since the time of the post-glacial high sea level, well before Neolithic times.

As it does not point exactly at the solstice, the calendrical function of the main alignment had to remain speculative, unless another clearly-indicated and reasonably precise alignment could be identified nearby; this could serve as a double check on any solar calendar and would tend to confirm that one was in use. The most probable candidate for a second alignment was the fallen stone on the ridge above Oak Bank, ~ 240 m north-west of the main alignment.

An attempt in 1981 to discover whether the Oak Bank stone could have been an artificial foresight to mark the midsummer sunset, as seen from the large terrace; resulted in the discovery, as a potential backsight, of the small stone North Platform a few metres north of the main outcrop and in the appropriate position; excavations in 1982, however, showed it to be relatively modern. It then seemed appropriate to test whether the fallen stone on Oak Bank might itself have been the backsight for an alignment.

One of us (P.F.G.) had found previously two unusual rock carvings a few metres south-east of the fallen Oak Bank stone, among the dense firs of a Forestry Commission plantation. Each consists of a pecked cup-mark of standard early Bronze Age type, but with a straight shallow pecked groove running through it (Fig. 3). Carving no. 1 has a groove ~ 60 cm long, on an azimuth of $130.5/310.5^{\circ}$, and points directly back at the Main Outcrop below. Carving no. 2 has a groove ~ 40 cm long on an azimuth of $\sim 81/261^{\circ}$, $\sim 2.1^{\circ}$ greater than the line defined by the two cup-marks themselves. These directions can be determined to within $\sim 0.5^{\circ}$ using a prismatic compass checked against a line of known azimuth.

The many fir trees around the carvings obscure the western and northwestern horizons; the groove of no. 1, however, was found to point at a featureless horizon several degrees to the right of the midsummer sunset position. The near east-west orientation of no. 2 suggested that it might mark an equinoctial line, the backsight for which would be the fallen stone nearby, but the eastern horizon across the loch is flat and featureless. Nothing could be seen of the western horizon at first until the azimuth of the groove was transferred 20 m to the base of the fallen stone; it then pointed almost exactly at a deep V-shaped notch ~ 1 km away in Siaradh Druim, the 'western ridge'. A metal rod laid in the groove of the rock carving confirmed, after some clearance of branches, that it points at this notch. This notch has an azimuth of $261^{\circ}32.5'$ and a declination of $+0^{\circ}08'$ as seen from the stone (perhaps $+0^{\circ}04'$, if the trees on the left slope are removed) (Fig. 4).

This position is within the range of the declinations of equinoctial sites identified by Thom, the mean of which is approximately at the 'megalithic equinox' of $+0.51^{\circ}$ (ref. 4). This is the date that would have resulted if the equinox was defined by counting the days in the year and sub-dividing (the summer half being a few days longer because of the eccentricity of the Earth's orbit (see p. 109 in ref. 4)). The Oak Bank alignment could have defined this megalithic equinox very well, if the Sun's lower limb served as the marker against the horizon. Figure 4 shows the $24'$ -wide zone of fluctuation—the amount

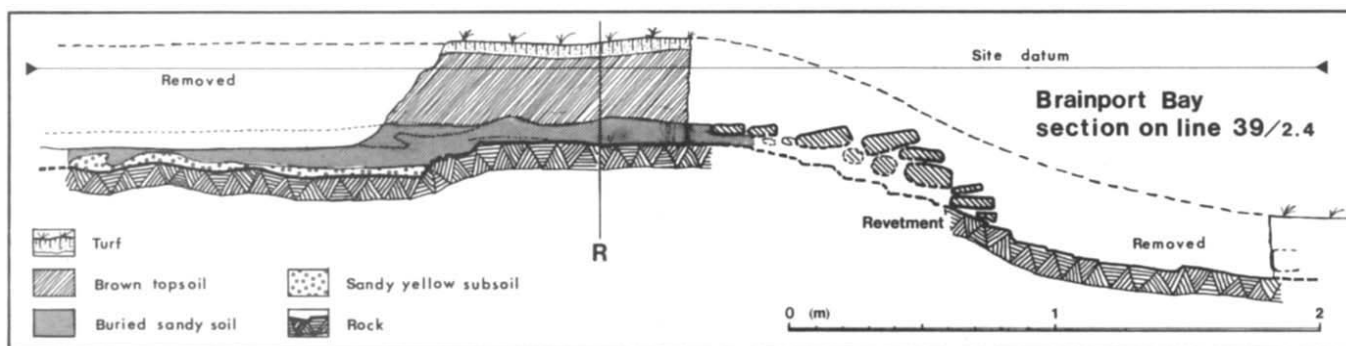


Fig. 2 Section of soil layers on the northwestern edge of the large terrace at Brainport Bay, showing the revetment of the terrace lying on the prehistoric ground surface.

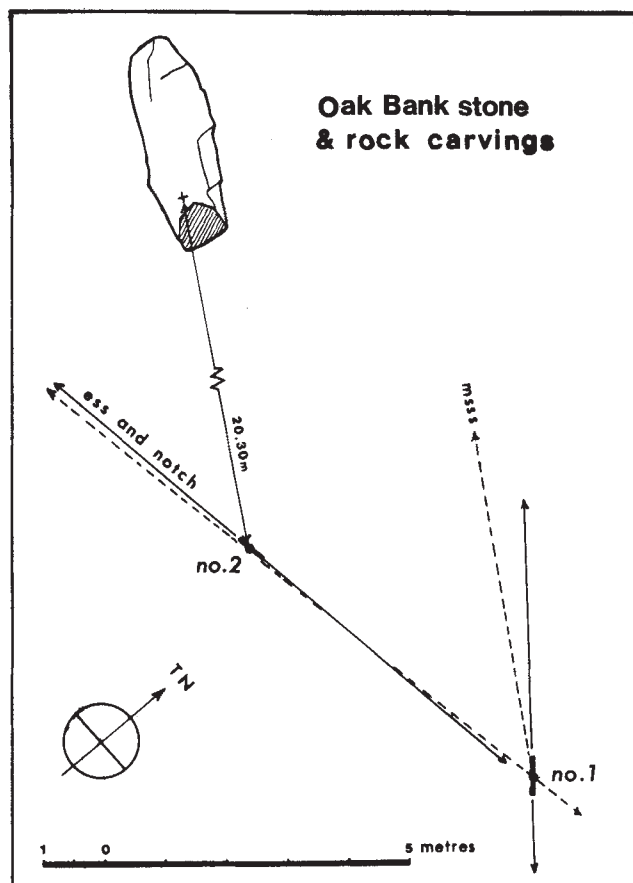


Fig. 3 Plan of artificial features on Oak Bank—the fallen stone and the two rock carvings. Carving no. 1 points back to the main site at a distance of 225 m. 'ESS' means equinoctial sunset and 'MSSS' midsummer sunset.

by which the solar declination can vary at sunset at the equinoxes—against the notch. Thus, the first appearance of the full disk in the notch in the spring, and the last in autumn, would have marked the day of the megalithic equinox and the alignment could have served as a good check on a solar calendar.

Ideas about the achievements of prehistoric man in Britain have focused traditionally on his technological and economic skills, deducible directly from the archaeological evidence; ceremonial and religious practices are much harder to recover but seemed to have been suitably primitive^{5,6}. However, much evidence from Thom shows that late Neolithic people erected standing stones to mark the position of the Sun and Moon (and some stars) on the horizon at important points of their cycles and that they thereby developed considerable astronomical expertise (see chapter 9 in ref. 4). These ideas have aroused considerable interest and controversy⁷⁻¹¹.

The particular aspect of the Thom hypotheses examined here is that many standing stones marked the 'backsights' (observers' positions) of alignments that could detect when the Sun was at the solstices, equinoxes and at other important intermediate dates of the solar calendar. These lines point towards 'foresights', which are natural marks on the horizon where the Sun rose or set at the times concerned and which are usually indicated in some way by the stones themselves.

The evidence assembled consists of a large number of standing stones, which could have been the backsights for such accurate alignments in Neolithic times, and of the statistical argument that this could not have happened by chance (ref. 12 and see chapter 8 and Fig. 8.1 in ref. 4). There has been much argument about whether such deliberately arranged astronomical lines (on which the more elaborate astronomical hypotheses developed by Thom (concerning the Moon) ultimately depend^{4,13,14}) were actually intended to be such by the standing stone erectors,

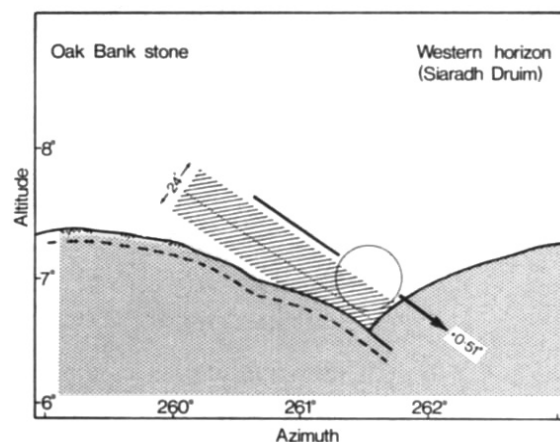


Fig. 4 Siaradh Druim, the 'western horizon', as seen from the Oak Bank stone with the Sun at the 'megalithic equinox'; the dotted line indicates where the real horizon may be, below the tree line. The shaded band shows the range of possible positions for the lower limb at sunset at that time.

about how accurate they were and for what exactly they were used¹⁰.

It can be argued that the genuineness, or otherwise, of the alignments will only be finally settled by testing a selection of examples on the ground, that is, by finding sites where one of the astronomical alignment hypotheses predicts the existence of some feature that can then be sought by excavation^{15,16}. One of us (E.W.M.) attempted to do this at the Kintraw standing stone in mid-Argyll, where an artificial platform was discovered, as required by Thom's theory that a precise midwinter sunset alignment existed there¹⁷. However, as the platform could not be dated, the evidence was inconclusive.

The results from Brainport Bay described here suggest that, at this site, there are two well-marked prehistoric solar alignments, the reality of which has been demonstrated beyond reasonable doubt and which could therefore be interlinked. The conspicuous line towards midsummer sunrise is formed by clearly artificial and by natural features and it has been dated as suitably early, directly, from associated artefacts and indirectly, from a ¹⁴C measurement. Yet the discovery that the 'observation boulders' are natural, implies that the alignment began as the chance finding of a suitable site with a long view to the north-east (presumably by people searching for one) and that features on it were modified to form a line towards the midsummer sunrise. This could explain why the 'foresight' mountains, though suitably distant, are not the ideal shape for marking the solstitial sunrise exactly; in other such cases, the Sun's upper limb rose or set along one slope, about parallel to the angle of its diurnal movement (see Fig. 12.2 in ref. 4).

This in turn suggests that the prominent north-east alignment was intended as much for ceremonial as for calendrical purposes; certainly the dawn spectacle is dramatic and could have been viewed by many people. It may also have been a useful calendrical alignment; the date of the solstice could have been found at any period in the past by halving the interval between successive risings in the notch. However, such an indirect alignment is less convincing statistically than one that points exactly at the solstitial declination, hence the search for a second solar line at the site.

Such a solar line, indicating the equinoctial sunset, was found at the Oak Bank stone rock carvings. Because of the obscuring fir trees it is also a good example of the discovery of a convincing alignment through a prediction made from Thom's general solar alignment hypothesis. On such independent testing of potentially accurate astronomical alignments as this depends surely the fate of ambitious re-assessments of the skill and organization of late Neolithic society in Britain¹⁸, and indeed that of the traditional views also^{5,6}.

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The first pre-Rhaetic therian mammal

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The earliest mammals in the fossil record are recognized by their multicusped and multiple-rooted cheek teeth, and in south-west Britain they appear abruptly in sediments that postdate the Rhaetic transgression at ~200 Myr. Here we report the discovery of teeth of the primitive mammal *Kuehneotherium* in a terrestrial fissure deposit from the Mendip Hills (Somerset, UK) that predates the Rhaetic transgression. The teeth were found in the sediment fill of a collapsed cave¹ at Emborough near Wells during the systematic reinvestigation of a number of late Triassic vertebrate sites. The assemblage is otherwise typically pre-Rhaetic and includes the gliding lizard *Kuehneosaurus*. The finding of mammalian remains throws doubt on the fundamental distinctiveness of pre-Rhaetic and post-Rhaetic vertebrate faunas.

Vertebrate-bearing fissure deposits are known at several sites in the Lower Carboniferous/Mesozoic unconformity either side of the Severn Estuary (Fig. 1). The fissures comprise a variety of features including karstic cave systems, tectonic joints and faults; their fills range from Triassic continental red marls to middle Jurassic marine limestones. In the first major review of the nature and occurrence of fissure deposits, Robinson¹ distinguished between Norian and Rhaeto-Liassic deposits which she believed could be recognized on the basis of their vertebrate faunas². The Norian fauna consisted entirely of sauropsids whereas the Rhaeto-Liassic faunas contained theropods and mammals in addition to sauropsids. There was also some vari-

ation in the nature of the sauropsid fauna, particularly with respect to archosaurs, which occurred only rarely in the Rhaeto-Liassic deposits. This broad division has been accepted by subsequent authors. Typical Norian assemblages include *Clevosaurus*, *Kuehneosaurus*, *Planocephalosaurus*, primitive crocodiles and pseudosuchians³⁻⁵, whereas the Rhaeto-Liassic forms include *Morganucodon*, *Kuehneotherium* and the lepidosaur *Gephyrosaurus*⁶⁻⁸.

More recently, Marshall and Whiteside⁹ have identified marine Rhaetic palynomorphs in sediments at Tytherington Quarry which contain a typical Norian sauropsid fauna, including *Clevosaurus* and *Planocephalosaurus*¹⁰; this work did not question the existence of two distinct faunas, but threw doubt on the validity of assuming an age prior to the Rhaetic transgression for the sauropsid assemblages. We have confirmed the presence of datable palynomorphs in the Tytherington sequence, but their usefulness is open to question as the deposits are contained within tectonically generated fissures which have suffered more than one phase of disturbance and mixing of the contents.

The Emborough deposit has yielded no palynomorphs and cannot be dated in this way, a problem in common with late Triassic continental sediments elsewhere¹¹. The deposit consists of a single large pocket of poorly sorted, locally derived limestone boulders and coarse conglomerate set in a matrix of red marl. The pocket has been interpreted as a collapsed cave deposit¹, and the conglomerate and marl components are typical pre-Rhaetic lithotypes; no Rhaetic or post-Rhaetic clastic components can be recognized. Further evidence of the pre-Rhaetic age of the Emborough deposit is provided by nearby outcrops of basal Rhaetic sediments which overlie a formerly planar transgression surface. This surface now rises southwards, and clearly extended across the top of the Emborough pocket.

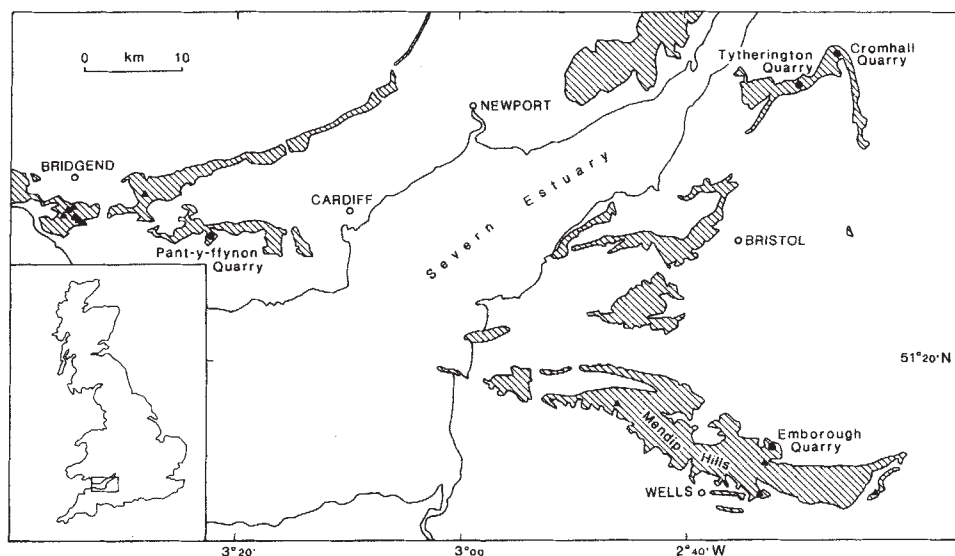


Fig. 1 Vertebrate-bearing Triassic fissure deposits either side of the Severn Estuary, UK. ▨, Carboniferous limestone outcrops; ●, localities mentioned in the text; ▲, other known localities.

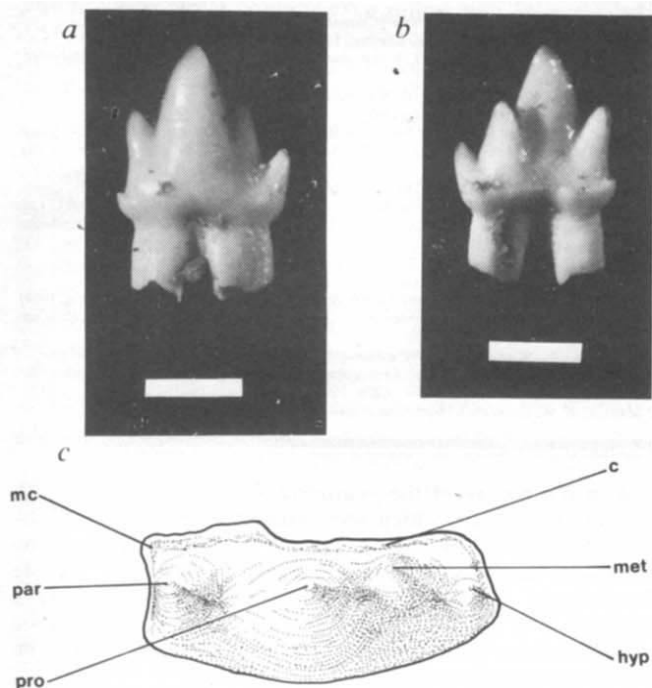


Fig. 2 Lower left molariform tooth of *Kuehneotherium* (AUP no. 11133). *a*, Buccal; *b*, lingual; and *c*, occlusal view. Scale bar, 0.5 mm. *c*, Cingulum; *hyp*, hypoconulid; *mc*, mesial cusplule; *met*, metaconid; *par*, paraconid; *pro*, protoconid.

Following Robinson¹, therefore, we are certain that the Emborough pocket is Norian in age.

The reptilian fauna of Emborough consists of lepidosaurs and archosaurs, and by far the most abundant of these is the gliding lepidosaurian, *Kuehneosaurus*¹²; this genus has also been identified at Cromhall⁴ and Pant-y-flyn¹³, although in much smaller numbers. Both of these localities are regarded as pre-transgressional in age^{4,14} and the genus is not known at the Rhaeto-Liassic localities.

We have confirmed an abundance of *Kuehneosaurus* at Emborough and have noted spheodontids. Of the two mammalian teeth, the best preserved (Fig. 2, AUP no. 11133) closely resembles a lower molar of *Kuehneotherium*, but with relatively low central cusps. Molariform teeth of *Kuehneotherium* were first described in detail by Kermack *et al.*⁷, who noted that in the lower molars the central cusps were generally elevated and higher than in the upper teeth. However, in one lower left molar described by Kermack *et al.*⁷ (BMNH C.855), the cusps are rather less elevated. The best-preserved Emborough tooth is practically identical in this and other respects, indicating beyond doubt that it belongs to the lower left mandible. The second Emborough tooth (Fig. 3, AUP no. 11134) is a premolar showing more polish than the first and it exhibits a well-defined wear facet. Kermack *et al.*⁷ emphasized that the premolars of the early mammals showed insufficient characters for a single tooth to be referable to a particular genus, citing the similarity of *Kuehneotherium* premolars to those of *Eozostrodon*. However, the occurrence of the distinct *Kuehneotherium* molar in the deposits indicates that in this instance the Emborough premolar is most probably also from *Kuehneotherium*.

This new record of *Kuehneotherium* from Emborough is important for two reasons. First, it is undoubtedly the earliest record of a therian and may even be the oldest fossil mammal. It predates the kuehneotheriid, *Woutersia*, and the Morganucodontid, *Brachyzostrodon*, described by Sigogneau-Russel¹⁵ from the French Rhaetic locality of Saint-Nicholas-de-Port. Although haramyid teeth have been described from earlier deposits, namely the Uppermost Middle Keuper of Halberstadt¹⁶, very little is known of this taxon. While they may be

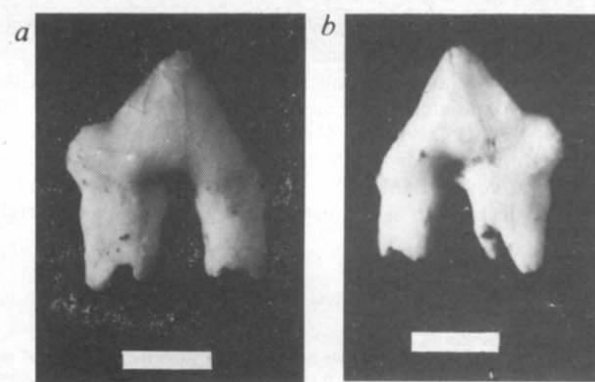


Fig. 3 A premolariform tooth of *Kuehneotherium* (AUP no. 11134). *a*, Buccal; and *b*, lingual view. Scale bar, 0.5 mm.

non-therian mammals, considerable doubt remains¹⁷⁻¹⁹ and their systematic position cannot be readily resolved within the framework of early mammals. From an analysis of the tooth wear facets of haramyids, it has been suggested that they may have had an antero-posterior movement of the lower jaw akin to that of the cynodont reptiles^{18,19}. Although this group of reptiles is widely accepted as being ancestral to the mammals, both prototherian and therian mammals have a lateral-medial motion of the lower jaw.

Second, the finding of a mammal in deposits with 'typically' Norian assemblages raises serious doubts about the validity of distinctions drawn between pre-transgression and post-transgression terrestrial vertebrate faunas. Although differences in the faunas can be recognized, these may now be no more significant than those between localities of the same age, or even between different sites at the same locality⁴.

It seems likely that ecological factors played an important part in determining these assemblages, in particular, feeding habits may have been a factor responsible for mammalian expansion at this time. From the nature of its dentition, *Kuehneotherium* was undoubtedly an insectivore^{6,20} but so too were several of its contemporary lepidosaurs^{4,8}. This raises the possibility of direct competition, but the reasons for the proliferation of the mammals in Rhaeto-Liassic times need not be related to this, nor were environmental factors connected with the Rhaetic transgression necessarily involved. By means of their implicit endothermy²¹, the early mammals such as *Kuehneotherium* may have avoided direct competition with other insectivores by adopting a nocturnal niche.

AUP and BMNH are abbreviations for Aberdeen University Palaeontology and the British Museum (Natural History) collections, respectively. We thank Dr D. J. Batten for palynological advice and Professor K. A. Kermack for his comments on the mammalian teeth. We also thank Mr J. Blatchford for allowing us access to Emborough Quarry. The work presented here forms part of a wider reinvestigation of late Triassic fissure faunas supported by NERC grant GR3/4419, which we gratefully acknowledge.

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Reduction of oak fecundity by low-density herbivore populations

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Explanations for the low levels of defoliation in natural woodlands^{1–6} have assumed that insect herbivores have a negligible effect on plant fitness and demography^{7,8}, perhaps because of plant compensation^{9–11}. Levels of defoliation between 5 and 15% are typical, and are attributed variously to the impact of natural enemies, inclement weather and low food quality in keeping herbivore populations at low densities^{12,13} (but see refs 14, 15). Here I report the results of a 4-yr study in which matched pairs of oak trees, *Quercus robur*, were sprayed regularly to exclude all herbivorous insects; despite the fact that the unsprayed trees lost only 8–12% of their leaf area, the sprayed trees consistently produced more seeds (from 4.5 to 2.5 times the numbers on unsprayed plants). It is not known whether this substantial reduction in seed production actually leads to a reduction in plant recruitment. It does seem, however, that plant compensation is not fully effective against low-density herbivore populations and, therefore, that slight differences in the level of defoliation between plants may cause important differences in plant fitness.

All invertebrate herbivores were excluded from six pairs of oak trees (matched initially for size, age and date of bud-burst, ranging in girth from 14 to 97 cm). I applied a mixture of contact (permethrin) and systemic (dimethoate) insecticides in a water base, and sprayed the control trees with an identical volume of water. The sprayed trees were further protected by a sticky band around the trunk which excluded walking insects such as the wingless females of the winter moth, *Operophtera brumata*. The sprays were applied monthly from March to September (but every 2 weeks in May and June), at a rate of between 1.5 and 5.5 litres per tree (~500 g active ingredient per hectare). There is no evidence of phytotoxic effects of the spray on the plants, nor is there any suggestion that these particular insecticides act

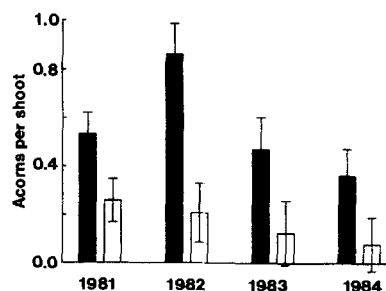


Fig. 1 Number of acorns per shoot on sprayed (solid bars) and unsprayed (open bars) trees in each of 4 yr. The data represent arithmetic means and 5% least significant differences, based on separate 2-way analyses of variance for each year (main effect means calculated over three size classes with two replicates in each class, using the same 12 trees each year).

as promoters of flowering or seeding in trees¹⁶. Regular counts using a variety of methods were made of insect herbivore numbers on nearby trees¹⁷.

The numbers of male and female flowers per shoot were assessed from a non-destructive sample of 100 randomly selected shoots per tree in June; defoliation levels, primary and lammas shoot lengths⁷ and acorn crop were assessed from a separate sample of 100 shoots in August each year (Table 1).

Insecticide treatment was effective in all years in reducing defoliation levels below 5% and in virtually eliminating gall-forming and sap-sucking insects. Reduced herbivore numbers had no effect on primary or lammas shoot growth, trunk-wood growth or flower production in the following year (Table 1), but led to a significant increase in acorn production (Fig. 1). A variable number of acorns on unsprayed trees were attacked by the gall-forming wasp, *Andricus quercuscalicis* (ref. 18 and R. S. Hails and M. J. C., in preparation). As seen in Table 1, the increase in acorn crop under spraying is not simply an artefact of excluding the gall-former.

In a separate study of 36 trees with girths ranging from 30 to 600 cm, there is no correlation between defoliation and acorn production either within trees (that is, from shoot to shoot) or between trees, in any year of this study. Failure to detect significant depression in fecundity results from the very high tree-to-tree variance in acorn production, and from the fact that certain trees are consistently the most heavily defoliated (the correlation between defoliation in year t and in year $t+1$ is significant for all pairs of years; $n=36$, $r>0.34$, $P<0.05$). Here, the failure to detect subtle ecological effects demonstrates the importance of manipulative field experiments in studies designed to elucidate the role of herbivores in plant population dynamics and as agents of natural selection.

Table 1 Insecticide spraying and reproductive performance in *Quercus robur*

Year Treatment	1981			1982			1983			1984		
	S	U	P	S	U	P	S	U	P	S	U	P
Defoliation, %	5.0	10.0	<0.01	3.0	8.0	<0.01	4.0	9.0	<0.01	3.5	11.5	<0.01
Wood increment, see below	0.56	0.54	NS	0.48	0.45	NS	0.39	0.32	NS	0.34	0.34	NS
Primary shoot, cm	11.1	8.4	<0.05	14.0	13.3	NS	11.3	11.8	NS	7.0	6.5	NS
Lammas shoot, cm	9.3	7.8	NS	14.8	14.6	NS	15.6	23.3	<0.05	2.7	4.4	NS
Male flowers, per shoot	0.41	0.44	NS	0.31	0.22	NS	0.39	0.36	NS	0.53	0.64	NS
Female flowers, per shoot	0.61	0.52	NS	0.48	0.33	<0.05	0.79	0.57	NS	0.72	0.69	NS
Peduncles, per shoot	0.30	0.19	NS	0.68	0.34	<0.05	<0.33	0.36	NS	0.32	0.13	NS
Acorns, per shoot	0.53	0.26	<0.05	0.86	0.21	<0.01	0.47	0.13	<0.05	0.36	0.08	<0.01
Acorns + galls, per shoot	0.53	0.26	<0.05	0.94	0.32	<0.01	0.47	0.40	NS	0.38	0.11	<0.05

Arithmetic means for six sprayed (S) and six unsprayed (U) trees divided into three size classes with two replicates per size class. Numbers estimated per shoot are from 100 shoots per tree each year. Wood increment is calculated from pre (t) and post ($t+1$) growing season measurements of girth (G cm) as $\ln(G_{t+1}^2) - \ln(G_t^2)$. Significance tests were based on separate 2-way analysis of variance for each year, with two levels of insecticide treatment (+ or 0) and three size classes of trees ($G < 25$, $25 \leq G < 50$, $G \geq 50$ cm); NS, not significantly different. Defoliation was caused principally by *Tortrix viridana* and *Operophtera brumata* but with many less important Lepidoptera. Peduncles measured in August include fruit stalks with acorns, galls, undeveloped fruits and unpollinated flower remains; not all peduncles bear sound acorns. Galls refer to the 'knopper galls' of *Andricus quercuscalicis*.

The results of the experiment described here are consistent with the hypothesis that oak is essentially a K-strategist^{19,20}, gaining in fitness by first ensuring its survival and by putting its carbohydrate and protein resources into seed production only when the needs of reserve-replenishment, wood growth, root growth and bud-filling have been met. Although several other studies of natural^{21,22} and artificial defoliation of trees^{23,24} have shown reduced seed production, these have involved typically much higher rates of defoliation (50–100%). The present study is the first to show that natural low levels of insect feeding can cause a significant reduction in seed set. As the number of female flowers produced in spring does not differ between the two treatments in most years, the increase in acorn production is seen to result from a reduction in the number of peduncles aborted and an increase in the average number of acorns filled

on each surviving peduncle. There is no evidence to suggest that insects feeding directly on the flowers or young acorns contribute to the observed result.

As there is no simple relationship between seed production in one year and seedling recruitment in the next⁹, we cannot be sure that these low levels of defoliation actually affect plant population density. This study does emphasize, however, that it is incorrect to assume that 'typical' low density populations of defoliating and sucking herbivores can have no impact on the demography of the tree. Also, given the great variation from tree to tree in average defoliation levels and the magnitude of the differences in fecundity observed in this experiment, these relatively scarce herbivores may act as potent agents of natural selection.

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Firing patterns of motor units in normal rats

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Skeletal muscles consist of motor units which may differ considerably in contractile properties and types of usage. Some units participate mainly in relatively rare, quick movements and contract rapidly and are easily fatigued (type FF); others contribute to the maintenance of posture and hence contract slowly and are fatigue-resistant (type S), while others are both fast and fatigue-resistant (type FR; ref. 1). Our understanding of motor control mechanisms and the dependence of contractile properties on usage would be enhanced if more quantitative information were available^{2–4} concerning the firing patterns of individual motor units during normal motor behaviour. Therefore, we have made continuous recordings for extended periods from single motor units in the fast extensor digitorum longus (edl) and the slow soleus (sol) muscle of freely moving adult rats. By counting the total number of discharges for each unit, and by determining the distributions of interspike intervals and the duration of the individual impulse trains, we have obtained information about firing rate, amount of use, modulation of muscle force and tonic and phasic behaviour for 16 motor units. We now report that these units fall into three classes apparently corresponding to type FF and FR in the edl muscle and type S in the soleus muscle.

Figure 1a–c shows representative discharges from two units in edl (a and b) muscle and one unit in soleus (c) muscle. The unit potentials were similar in shape and amplitude throughout the 24-h recording, and superimposing the traces indicated that they originated from one unit only (Fig. 1d–f). In Table 1 the units have been ordered according to the percentage of total time that they were active. Three classes of firing patterns can be discerned. In the first class (edl-1 units) the units were active for only 0.5–3 min per 24 h (0.04–0.22% of the total time). In the second class (edl-2 units) the total active time was 23–72 min

(1.6–5%), and in the third class (sol-1 units), 5.3–8.4 h (22–35%). The soleus units formed a separate class by firing more impulses at a lower frequency (~20 Hz) than any edl units. The two classes of edl units could not be clearly distinguished on the basis of firing rate alone, although, on the whole, edl-1 units fired at higher frequencies than edl-2 units (Fig. 2, Table 1). The edl-1 units, however, were by far the least active units, generating impulse trains that were always brief and in 70–80% of cases started with a pair of spikes having a short interspike interval (initial doublet of Fig. 1a, d). In some respects edl-2 units occupied an intermediate position in that they resembled edl-1 units by firing at high frequency and sol-1 units by generating a large number of impulses which sometimes occurred as long trains of impulses.

The firing pattern of soleus units was relatively uniform. There was some variation between units in amount of activity, but little variation in median firing rates. This pattern probably represents S-type motoneurons innervating slow, fatigue-resistant type I muscle fibres which make up the large majority of fibres in adult rat soleus muscle⁵. The edl muscle contains almost exclusively fast motor units⁶. When stained for myosin ATPase activity, approximately half the muscle fibres appear as type IIA and the other half as type IIB⁷, which, in general, correspond to FR and FF units, respectively¹. It seems likely, therefore, that the infrequently used edl-1 units represent FF units, whereas the much more frequently used edl-2 units correspond to FR units.

There exists evidence that motor unit characteristics are more continuously distributed than is suggested by Table 1 (refs 1, 8). The clear separation between soleus and edl units is not surprising given such widely differing muscles. Within the edl, however, a larger unit sample would probably have narrowed the gap between the two classes of firing patterns, and the existence of intermediate types cannot be excluded. Nevertheless, even within the three classes of firing patterns presented in Table 1, there is considerable variation in the number of discharges and in the frequency of spikes within each discharge—this variation may partly explain the variation in contraction speed and metabolic enzyme activity observed in different motor unit types and in type I, IIA and IIB fibre populations^{1,9,10}.

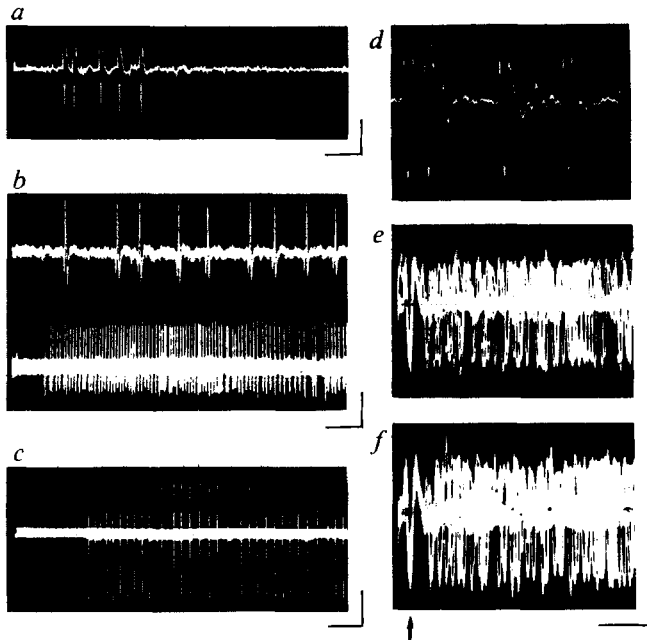


Fig. 1 Typical discharges for each of the three classes of motor units shown in Table 1: *a*, edl-1 unit; *b*, edl-2 unit; *c*, sol-1 unit. Traces *d-f* show that it is possible to record continuously for 24 h from one motor unit (in this case an edl-1 unit). Spontaneous action potentials trigger the oscilloscope trace and are displayed after a fixed delay (arrow) on a single trace (*d*) or on ~100 superimposed traces (*e* and *f*). The action potentials have a similar appearance at the beginning (*e*) and at the end (*f*) of the 24-h recording. Note that there is also a minimum spike interval after the first triggered spike in each of the traces in *e* and *f*, which makes the presence of more than one unit highly unlikely. Vertical bars represent 0.5 mV and horizontal bars 20 ms (*a*, *b*), 200 ms (*b*, *c*) and 10 ms (*d-f*). The motor unit potentials were similar in amplitude (0.5–1 mV) to fibrillatory potentials recorded from the same electrodes 2–3 days after cutting the sciatic nerve, indicating that the potentials were generated by single muscle fibres close to the recording electrodes.

Methods. Adult male Wistar rats were anaesthetized with chloral hydrate and pentobarbital (Equithesin, 0.4 ml per 100 g). Four Teflon-coated platinum wires (outer diameter 0.25–0.50 μ m), soldered to the ends of four flexible Teflon-coated multi-stranded steel wires, were guided by a thin needle and tube longitudinally into the centre portion of the edl or sol muscle. The distance between each cut end was ~100 μ m. The steel wires continued under the skin, through an attachment fixed to the skull by screws and dental cement, into a flexible protective tube which connected the rat to rotating contacts and preamplifiers above it. Muscle action potentials were recorded between the ends of the two wires that gave the best spike isolation and signal-to-noise ratio. The action potentials were stored on tape, and reproduced later on a storage oscilloscope for examination of the entire 24-h recording. Only potentials that could be attributed confidently to the same unit for the entire 24-h period were accepted for further computer analysis. The recordings were usually best 2–3 days after implantation; thereafter, the potentials often declined in amplitude, but with occasional improvements. Such changes made it difficult to be sure that we were recording from the same unit for periods much longer than 24 h. From the time of implantation the rats were kept in the laboratory in large buckets where they moved freely, were more active during the night and showed apparently normal behaviour in terms of movement, feeding and sleeping.

If we are justified in assigning the firing patterns of edl-1, edl-2 and sol-1 units to FF, FR and S-type motor units, some interesting relations between firing pattern and contractile properties appear. First, low firing frequency characterizes slow motor units, whereas high firing frequency characterizes fast motor units. Second, motor units that are used frequently are more fatigue-resistant than those used infrequently. Third, motoneurons fire naturally at frequencies that give maximum control over the tension developed by the muscle fibres. Figure

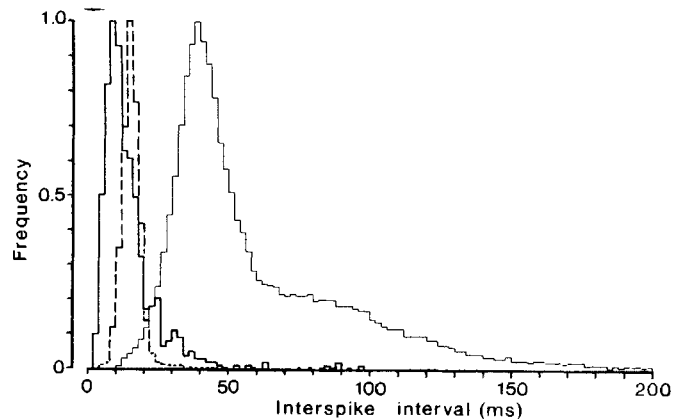


Fig. 2 Normalized frequency distribution of the interspike intervals obtained during a 24-h recording from the units shown in Fig. 1 *a-c*. The thick solid line shows the distribution for the edl-1 unit; broken line, the edl-2 unit; and the thin continuous line, the sol-1 unit. The histograms contain, respectively, 686, 1,223 and 91,097 intervals and are typical of the three classes of motor units shown in Table 1. Intervals longer than 200 ms, representing only a very small fraction of the total, have been omitted. From such histograms, an impulse train was defined as starting (and stopping) when the duration of the preceding (and subsequent) intervals was at least 200 ms for edl units and 500 ms for sol units; this definition ensured that the tension produced by any preceding impulse declined to zero before another train was said to start.

3*a* shows the tension developed by stimulating soleus and edl muscles at different frequencies. In soleus muscle twitches begin to fuse at ~8 Hz and maximum tetanus is reached at ~100 Hz. In edl, similar force summation occurs between 30 and 200 Hz. The natural firing rates of soleus and edl units (Fig. 3*b*) show a striking correspondence to the rising portions of their respective tension-frequency curves (Fig. 3*a*), which cover the frequencies over which muscle force can be effectively modulated.

These relationships support recent evidence that the pattern of evoked muscle activity has an important role in the regulation of speed of contraction and fatigue resistance^{11,12}. Thus, a large amount of low-frequency (10 Hz) stimulation of the nerve induces both slow contraction speed and high fatigue resistance in fast muscles^{13,14}. Conversely, the slow soleus muscle contracts much faster when denervated and stimulated at high frequency (ref. 15 but see also ref. 11) and becomes less fatigue-resistant if the amount of stimulation is kept low¹⁶. Similar, but less pronounced, speeding up occurs if the nerve to the soleus is intact and the muscle continues to show tonic low-frequency activity in between the high-frequency stimulus trains (T.L. and R.H., unpublished). Mechanisms appear to exist, therefore, whereby the contractile properties of muscle fibres can be adjusted continually to the pattern of afferent impulse activity. Thus, effective performance can be maintained throughout life despite changes in the level and pattern of activity.

Certain features of the edl-1 firing pattern deserve special mention. First, edl-1 units rarely generated more than five or six impulses at a time: a single impulse was by far the most common event, two impulses per burst the next common event and so on. By varying the number of stimuli from 1 to 5 in a train of stimuli to the edl muscle, we found that the force output can be varied between ~15% and 80% of maximal tetanic tension. Hence, the force output of the motor unit must normally show considerable variation as a result of the observed variation in number of motoneuron discharges per burst. Second, although the total number of impulses in edl-1 units was 1–2 orders of magnitude lower than in edl-2 or sol-1 units, the edl-1 units nevertheless discharged trains of impulses on hundreds or thousands of occasions per day. Assuming that the edl-1 units are FF units, this suggests that FF units participate not only in relatively rare, high-force quick movements such as jumping,

Table 1 Firing patterns of individual motor units in the rat

Motor unit	Amount of activity		Burst characteristics			
	% Time occupied by bursts	No. of impulses per day	First interval (Hz)	All intervals (Hz)	No. of impulses per burst	Longest duration (s)
edl-1	0.04	2,600	250 (333-167)	91 (111-58)	3 (1-18)	0.83
	0.07	4,600	167 (333-125)	83 (111-67)	3 (2-16)	1.15
	0.09	5,600	143 (200-77)	77 (91-67)	13 (3-35)	3.9
	0.14	7,800	125 (200-77)	77 (83-53)	5 (2-12)	1.1
	0.22	11,200	91 (167-63)	67 (77-50)	4 (1-8)	1.15
edl-2	1.6	105,500	71 (125-46)	83 (100-67)	3 (1-11)	59
	2.0	89,500	50 (100-40)	53 (59-50)	25 (3-65)	120
	2.6	119,200	43 (56-30)	56 (67-40)	6 (2-18)	141
	3.5	139,400	36 (44-26)	48 (53-42)	4 (2-13)	89
	5.0	243,100	41 (67-29)	58 (67-53)	39 (6-56)	135
sol-1	22	309,500	13 (23-7)	21 (26-12)	5 (1-35)	300
	29	449,500	14 (24-6)	19 (26-14)	8 (1-39)	390
	31	453,400	14 (29-6)	19 (29-14)	7 (1-38)	548
	31	446,400	14 (25-6)	19 (23-13)	9 (1-35)	290
	32	482,500	15 (31-5)	21 (26-15)	10 (1-40)	510
	35	495,800	14 (23-6)	18 (25-13)	7 (1-36)	490

Columns 4 and 5 give median values for the instantaneous frequencies for certain intervals in all the bursts generated by one motor unit in one day. Column 4 refers to the first interval and column 5 to all intervals in each burst. Note that only in the edl-1 units is the frequency highest at the onset of a burst, in agreement with the frequent occurrence of initial doublets in these units (Fig. 1a, d). Numbers in parentheses are quartile values.

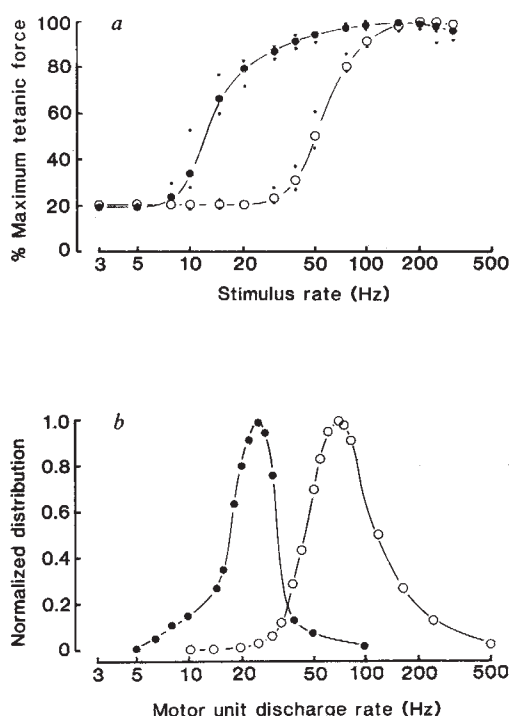


Fig. 3 Correspondence between tension-frequency curves (a) and motor unit firing rate (b) in soleus (●) and edl (○) muscles. The tension-frequency curves were obtained in acute experiments *in situ*, at 35 °C and at optimal muscle length by stimulating supramaximally the nerves to normal soleus and edl muscles. Each symbol represents the median isometric tension obtained from eight (sol) or six (edl) muscles and represents the highest tension produced regardless of when this occurred during a 1-s stimulus train at the frequencies indicated. Total ranges that extend beyond the symbols are indicated by dots. The curves in b were obtained from histograms of the type shown in Fig. 2 for all the units shown in Table 1. Each histogram was divided into segments of 5-ms duration, and the number of interspike intervals within each segment were counted, converted to frequency by taking the reciprocal values and expressed relative to the most commonly occurring frequency for all 6 units in soleus muscle and 10 units in edl muscle.

but also in more frequent, brief, inconspicuous contractions, possibly of a corrective nature, such as those described for high threshold units in humans⁴. The total amount of activity in edl-1 units, expressed as average firing rate over ≥ 1 day, was only 0.03–0.13 Hz (Table 1). Even lower mean impulse rates prevent supersensitivity to acetylcholine and other abnormal responses in denervated muscles if the activity is evoked relatively often by direct stimulation, for example, by a brief 100-Hz stimulus train applied every 2 h (mean frequency 0.01 Hz; refs 16, 17 and unpublished observations). In contrast, denervated organ-cultured muscles, which fibrillate relatively intensely for ~ 20 h, then remain silent for 2–3 days before a new cycle is repeated, show much higher average firing rates (~ 1 Hz) and yet become both atrophic and supersensitive to acetylcholine¹⁸. This suggests that timing, rather than the amount of activity, is important for the maintenance of normal muscle function. The relatively frequent activation of edl-1 units may explain, therefore, why FF units retain normal properties despite high activation thresholds¹⁹ and low total amounts of activity.

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Stimulation of food intake in rats by centrally administered hypothalamic growth hormone-releasing factor

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Hypothalamic growth hormone-releasing factors (GRFs) have been purified recently from human pancreatic (hp) tumours^{1,2} and from rat hypothalamus (rh)³. GRF peptides have strong homology with peptides of the glucagon, vasoactive intestinal polypeptide and PHI-27 family. Aside from their potent actions on release of somatotropin, no other biological actions of GRFs have been reported. GRF has been localized in neurones bordering the ventromedial hypothalamic nucleus (refs 4, 5; L. Swanson and P. Sawchenko, manuscript in preparation), a region associated frequently with experimental analysis of feeding behaviour⁶. We now report that intracerebroventricularly (i.c.v.)-administered rhGRF and hpGRF(1-40) in doses of 0.2, 2.0 and 20.0 pmol, produced an increase in food intake in hungry rats. This effect seemed to be specific to GRF as i.c.v. injections of a structurally related but physiologically inactive peptide⁷ in the same doses had no effect on feeding. In addition, peripheral injections of rhGRF or growth hormone had no effect on food intake, suggesting that the present effects may be mediated centrally. Injections (i.c.v.) of rhGRF (0.2, 2.0 and 20.0 pmol) had no effect on general activity, suggesting that GRF does not produce nonspecific arousal.

Thirty-two male Wistar rats (250–300 g) were used and a 23-gauge guide cannula was implanted 1 mm dorsal to the right or left lateral ventricle of each rat (coordinates for the surgery using the Pellegrino and Cushman atlas⁸: 0.6 mm posterior to bregma, 2.0 mm lateral of the midline suture, 3.2 mm ventral of the dorsal surface of the skull). Rats were then returned to their home cages where they had free access to food and water and were allowed 1 week to recover from surgery. On the eighth day after surgery (day 1), rats were started on a food deprivation schedule consisting of depriving the rats of food for 23 h and then placing them in individual wire testing chambers (20 × 25 × 36 cm) where they had access to wet mash [ratio of water/food 7:5 (v/v)] for 30 min. Food intake during this period was determined by calculating the difference between pre- and post-test food weight (including spillage). Rats were then returned to their home cages and allowed a further 30 min access to their regular laboratory diet after which the food was removed, but water was still available throughout the 23 h of food deprivation. This procedure was repeated for 5 days; by day 5, rats had stabilized their food intake during the 30-min test session ($\pm 10\%$ of average of the last 2 days). On day 6, all rats were pretreated 30 min before the feeding test session with one of four doses of rhGRF (0.0, 0.2, 2.0 or 20.0 pmol) administered i.c.v. All injections were made in a 2- μ l volume of 0.01% ascorbic acid with a 30-gauge injector using gravitational force. Rats were maintained on the deprivation schedule following the day 6 test session and again tested for food intake the following day. In a second experiment, 26 different rats were tested with hpGRF(1-40) for food intake using the same method.

Both rhGRF and hpGRF(1-40) had a stimulatory effect on food intake. Figure 1 shows that although there was little change in the amount of food intake in rats treated with vehicle alone, there was an increase in intake for hpGRF(1-40)- and rhGRF-

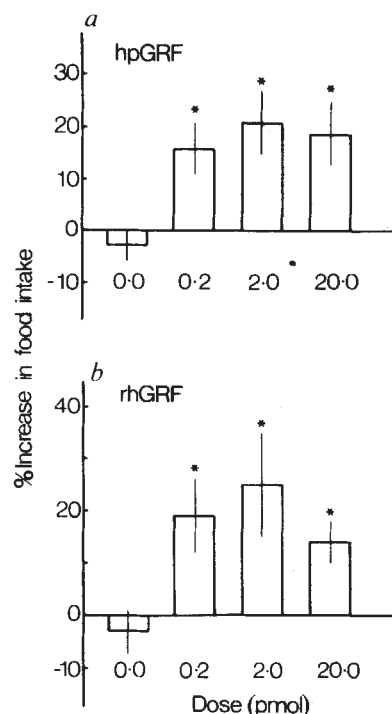


Fig. 1 The mean per cent increase in food intake after i.c.v. injections of a, hpGRF(1-40) ($n=7$ for vehicle and 2.0-pmol groups, $n=6$ for 0.2- and 20.0-pmol groups) and b, rhGRF ($n=8$ for each group). 0.2, 2.0 and 20.0 pmol correspond to 1.0, 10.0 and 100.0 ng, respectively. Rats were assigned to their respective drug condition on the basis of their food intake on the previous 2 days such that the mean food intake before GRF treatment was approximately the same for all four drug treatment groups. Error bars represent the s.e.m. * Significantly different from vehicle-treated group, $P<0.05$, Duncan multiple range *a posteriori* test.

treated rats. A one-way analysis of variance revealed that this effect is statistically significant for both the hpGRF(1-40)-treated rats ($F(3, 22) = 4.398$, $P = 0.014$) and the rhGRF-treated rats ($F(3, 28) = 3.159$, $P = 0.039$). (All individual group comparisons were carried out using the Duncan multiple range *a posteriori* test.) Individual group comparisons with vehicle-treated rats revealed significant increases in food intake at 0.2, 2.0 and 20.0 pmol for both the hpGRF(1-40)-treated rats ($P<0.05$) and the rhGRF-treated rats ($P<0.05$). The mean food intake ($\pm$ s.e.m.) on control days was 21.3 ± 2.1 g for rhGRF-treated rats and 28.5 ± 2.2 g for hpGRF(1-40)-treated rats. Both hpGRF(1-40)- and rhGRF-treated rats returned to baseline levels of food intake on the day after GRF tests: $F(3, 22) = 0.387$, not significant (NS) and $F(3, 28) = 0.720$, NS, respectively.

In a third experiment using the same procedure, 24 rats were tested with GRF(3-40), a structurally related peptide completely unable to stimulate growth-hormone release⁷, as a control for possible nonspecific effects of i.c.v. GRF injections. There were no effects of GRF(3-40) on food intake ($F(3, 20) = 1.007$, NS), suggesting that the facilitation in food intake observed in experiments 1 and 2 was probably not due to nonspecific effects of i.c.v. peptide administration.

Rats were maintained on the deprivation schedule following GRF(3-40) testing to investigate whether the increased food intake reflected an increased release of adenohypophyseal growth hormone or a direct result of peripheral responses that might also affect feeding. Thus, the same rats used for GRF(3-40) tests were re-divided into three groups and tested with rhGRF (0, 2.0 or 200.0 pmol) on day 8 by intraperitoneal (i.p.) injection in a 0.4-ml volume of 0.01% ascorbic acid. The peripheral injections of rhGRF had no significant effect on food intake ($F(2, 21) = 0.637$, NS). An additional six rats were subsequently tested with 0.2 pmol rhGRF injected i.p. Consistent

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with the effects of 2 and 200 pmol, 0.2 pmol of rhGRF injected i.p. had no significant effect on food intake, $F(5) = 0.7$, NS. The fact that peripheral rhGRF did not influence food intake suggests that the GRF-induced facilitation of feeding is due neither to increased growth-hormone release nor to actions of GRF directly in the periphery.

To examine further the possibility that the present facilitatory effects of GRF on feeding are due to peripheral actions of GRF or increased growth-hormone release, the effects of peripheral injections of rat growth hormone were tested by the same procedure as experiments 1 and 2. On day 6, 24 rats were pretreated 30 min before the feeding session with one of four doses of rat growth hormone (0, 10, 50 and 250 μg per rat) administered i.p. These doses were chosen based on findings indicating that growth hormone values may normally reach $2 \mu\text{g ml}^{-1}$ in rat blood following endogenous GRF secretion (W.V., unpublished data). All injections were made in 0.4 ml of 0.03 M NaHCO_3 in 0.15 M NaCl and six rats were assigned to each growth hormone dose.

Our results demonstrated that rat growth hormone had no significant effect on food intake ($F(3, 20) = 0.313$, NS). The mean per cent control food intake ($\pm$ s.e.m.) after 0-, 10-, 50- and 250- μg treatments was 104 ± 10 , 98 ± 2 , 100 ± 7 and 95 ± 3 , respectively. The mean food intake ($\pm$ s.e.m.) on control days was 25.8 ± 1.1 g, suggesting that in the present conditions, the GRF-induced facilitation in food intake was probably not due to an increase in growth hormone release.

We tested the hypothesis that the increases in feeding observed following i.c.v. GRF treatment are the result of the general behavioural activating properties of GRF. Thus, 24 rats were tested for their locomotor response to one of the following doses of rhGRF: 0.0, 0.2, 2.0 or 20.0 pmol. rhGRF was administered i.c.v. as in experiment 1 and six rats were tested with each dose. Locomotor activity was measured as in previous studies⁹. We found no significant group effect of rhGRF on locomotor activity ($F(3, 20) = 0.399$, NS). Mean photocell beam interruptions ($\pm$ s.e.m.) for the 0-, 0.2-, 2.0- and 20.0-pmol doses of rhGRF were 334 ± 59 , 438 ± 87 , 260 ± 50 and 392 ± 94 , respectively. These results suggest that rhGRF, in the doses which produced a stimulation in food intake in the present study, do not produce general behavioural activation.

Our finding that low doses of centrally injected GRF can stimulate feeding is the first demonstration of a behavioural role for GRF. It is not yet clear whether the appetitive behaviour promoted by GRF is involved functionally in other peripheral actions initiated by central GRF secretion. Thus, the classic effects of growth hormone to promote growth of soft tissue in bone and cartilage¹⁰, together with the central appetitive effect of GRF, suggest that central and peripheral factors may collectively underly growth processes in an integrated manner. Citing the actions of luteinizing hormone-releasing hormone, Moss and McCann¹¹ have proposed previously the concept of a central/peripheral integration of neuropeptide function. In the present study we add the actions of GRF as another possible case in point.

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Co-localization of GABA_A receptors and benzodiazepine receptors in the brain shown by monoclonal antibodies

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The most abundant inhibitory neurotransmitter in the central nervous system, γ -aminobutyric acid (GABA), exerts its main effects via a GABA_A receptor that gates a chloride channel in the subsynaptic membrane¹. These receptors can contain a modulatory unit, the benzodiazepine receptor, through which ligands of different chemical classes can increase or decrease GABA_A receptor function^{2,3}. We have now visualized a GABA_A receptor in mammalian brain using monoclonal antibodies. The protein complex recognized by the antibodies contained high- and low-affinity binding sites for GABA as well as binding sites for benzodiazepines, indicative of a GABA_A receptor functionally associated with benzodiazepine receptors. As the pattern of brain immunoreactivity corresponds to the autoradiographical distribution of benzodiazepine binding sites, most benzodiazepine receptors seem to be part of GABA_A receptors. Two constituent proteins were identified immunologically. Because the monoclonal antibodies cross-react with human brain, they provide a means for elucidating those CNS disorders which may be linked to a dysfunction of a GABA_A receptor.

Monoclonal antibodies were raised in mice immunized with a highly purified GABA_A receptor/benzodiazepine receptor fraction from bovine cerebral cortex⁴, a preparation characterized by: (1) the presence of binding sites for benzodiazepines as tested by ³H-flunitrazepam (³H-FNZ) binding; (2) high-affinity binding sites for GABA, assayed by ³H-muscimol binding; and (3) low-affinity binding sites for GABA, monitored by the allosteric enhancement of benzodiazepine receptor affinity in micromolar concentrations of GABA⁴. The ligand specificity of these binding sites corresponds to that of the GABA_A receptor/benzodiazepine receptor complex (receptor complex) in synaptic membranes⁵. The presence of low-affinity GABA binding sites coupled to benzodiazepine receptors is of interest as, electrophysiologically, the receptor complex is activated by micromolar concentrations of GABA⁶⁻¹⁰. We obtained a total of 22 monoclonal antibodies, with which 4 different epitopes of the GABA_A receptor complex could be distinguished. We describe here two antibodies (bd-17 and bd-24) representative of the interaction with two of the receptor epitopes.

Antibodies recognizing the receptor complex would be expected to immunoprecipitate with a compound having the binding characteristics of the purified receptor. To test this premise, a bovine receptor complex fraction⁴ was incubated with the antibody bd-17 or bd-24, followed by precipitation with goat/anti-mouse immunosorbent. As neither antibody inhibits the binding of ³H-FNZ or ³H-muscimol, the resuspended immunosorbent beads were tested in radioligand binding assays (Fig. 1). In controls without bd-17 or bd-24, no binding of ³H-FNZ or ³H-muscimol was found. When increasing concentrations of either bd-17 or bd-24 were added, however, increasing amounts of the binding sites for ³H-FNZ and ³H-muscimol were recovered, ≈ 90 –95% of the total activity (Fig. 1). Binding of the benzodiazepine ³H-diazepam (5 nM, 90 min, 4 °C) to the pellets was increased by $50 \pm 5\%$ when tested in the presence of 10 μM GABA, indicating the presence of low-affinity GABA sites. The ligand specificity of these sites corresponds to that of membrane-bound GABA_A receptors as benzodiazepine receptor binding was also enhanced by muscimol (1 μM), 3-amino-propanesulphonic acid and tetrahydroisoxazopyridinol (THIP, 10 μM), but not by the GABA_B receptor agonist

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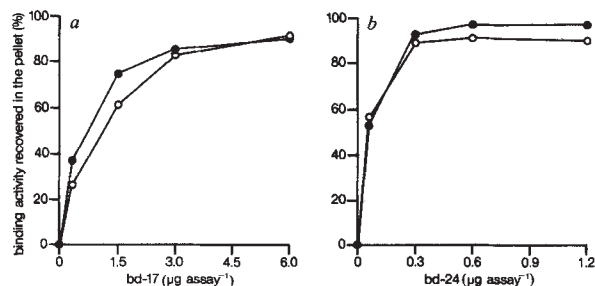


Fig. 1 Immunoprecipitation of binding sites for benzodiazepines and GABA with two monoclonal antibodies (*a*, bd-17; *b*, bd-24), recognizing different receptor epitopes. Filled and open circles represent specifically bound ^3H -FNZ and ^3H -muscimol, respectively.

Methods. A partially purified GABA_A receptor/benzodiazepine receptor preparation from bovine cerebral cortex (100 μl , $\sim 30 \mu\text{g ml}^{-1}$; ref. 4) was incubated for 2 h at 4°C with 200 μl of bd-17 or bd-24 (previously purified by affinity chromatography on a sheep/anti-mouse antibody column), respectively. 200 μl immunosorbent (goat anti-mouse immunoglobulin G-agarose, Sigma A-6531, in phosphate-buffered saline (PBS), 0.2% Triton X-100 at a v/v ratio of 1:1) were added and the incubation continued for another $2\frac{1}{2}$ h in the presence of 1% bovine serum albumin (BSA). After centrifugation, the pellet was washed once in 1 ml cold PBS, 0.2% Triton X-100, 1% BSA. The first supernatant and the final pellet were subjected to separate radioligand binding assays with ^3H -FNZ (10 nM, 60 min) and ^3H -muscimol (100 nM, 30 min). The radioligand binding assay for the supernatant was as described previously²¹. Radioligand binding to the pellet was performed in an Eppendorf tube on a turning wheel at 4°C in 1 ml (final volume) of assay buffer (50 mM Tris/citrate pH 7.1 + 0.2% Triton X-100) containing the radioligand. After a short centrifugation, the resuspended pellet and rinse fluid of the tube were transferred onto a glass fibre filter and washed with $2 \times 5 \text{ ml}$ cold buffer. The radioactivity retained on the filter was measured using an external standard procedure. For each ^3H -FNZ and ^3H -muscimol binding experiment, nonspecific binding was determined in a parallel assay in the presence of $3 \mu\text{M}$ diazepam or 1 mM GABA, respectively. In controls which contained either no or an irrelevant antibody, only 7% of the binding activity was recovered in the pellet. This nonspecific precipitation was subtracted from all values. The results are expressed as % of total activity (100% = 100,000 d.p.m. ^3H -FNZ and 44,000 d.p.m. ^3H -muscimol, respectively); mean values of two experiments which differed $< 10\%$ are given. Based on antibody concentration, the antigen-binding efficiency of bd-24 was about 10-fold higher than that of bd-17.

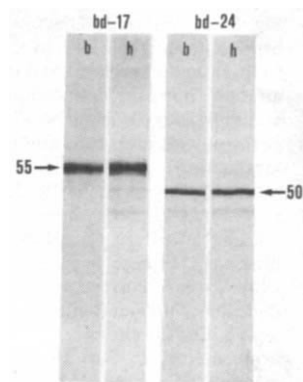
baclofen (100 μM). Thus, the immunoprecipitated material contained binding sites for benzodiazepines as well as low- and high-affinity binding sites for GABA, indicating that the antibody recognized the receptor complex. The same results were obtained with a purified receptor preparation from postmortem human cerebral cortex.

The presence of benzodiazepine receptors in the immunoprecipitated pellet was confirmed further by identifying the receptor protein with a covalently bound radioligand. When a purified bovine receptor sample was photoaffinity labelled with ^3H -FNZ (10 nM, 5 min ultraviolet; ref. 11) and immunoprecipitated subsequently with saturating amounts of either bd-17 or bd-24, 90% of the radioactivity incorporated into the protein was recovered in the pellet.

Benzodiazepine receptors in rat brain membranes consist of subunits of relative molecular mass (M_r) 50,000 and 55,000 (refs 11, 12). Similarly, a highly purified receptor complex from bovine brain contains two subunits of M_r 50,000–53,000 and 55,000–57,000, respectively^{4,13–15}. We therefore expected these proteins to be recognized by the antibodies bd-17 and bd-24 in immunoblot experiments. In receptor preparations from post-mortem human or bovine cerebral cortex, bd-24 labelled specifically a protein of M_r 50,000 and bd-17 a protein of M_r 55,000 (Fig. 2). Thus, each antibody apparently interacted with only one of the two putative constituent proteins of the receptor complex (Fig. 2). Both antibodies, however, were able to

Fig. 2 Immunoblot of a bovine (b) and human (h) receptor complex preparation, showing the specificity of bd-17 and bd-24 for proteins of M_r 55,000 and 50,000, respectively.

Methods. Partially purified receptor complex preparations from bovine or human cerebral cortex⁴ were subjected to SDS-polyacrylamide gel electrophoresis. The resolved polypeptides were electroblotted onto a nitrocellulose membrane (HAWP, 0.45 μm pore size, Millipore) at 1 A for 90 min at room temperature in 25 mM Tris, 192 mM glycine and 20% (v/v) methanol, pH 8.3, according to Towbin *et al.*²². Unreacted sites on the nitrocellulose were blocked by three 10-min washes in 0.05% Tween-20 in PBS (PBS-Tween), according to De Blas and Cherkowski²³. Strips of nitrocellulose were then incubated in bd-17 or bd-24 hybridoma supernatants, containing 0.05% Tween-20, at room temperature overnight under rocking. After three 10-min washes with PBS-Tween, the strips were incubated with ^{125}I -labelled sheep anti-mouse immunoglobulin ($0.5\text{--}1.0 \times 10^6$ c.p.m. ml^{-1} ; 30–60 ng ml^{-1}) in PBS-Tween, for 3 h at room temperature under rocking. Affinity purification and iodination of sheep anti-mouse immunoglobulins has been described previously²⁴. Membrane strips were then washed $4 \times$ for 20 min in PBS-Tween and once in H_2O , dried and exposed to Kodak X-Omat AR film, using an intensifying screen (DuPont). M_r values of 50,000 and 55,000 are indicated by 50 and 55 respectively. The immunoreactive minor bands were presumably degradation products. All postmortem human brain was obtained through the Institutes of Pathology, Kantonsspital, Liestal and Basel (Switzerland).



immunoprecipitate the entire receptor population (Fig. 1), which suggests that the receptor complex has a mixed subunit composition. Furthermore, the immunoblot pattern (Fig. 2) excludes a simple proteolytic precursor-product relationship for the two proteins. No immunoreactivity was discovered in immunoblots using peripheral organs; when crude solubilizes of rat and bovine liver, heart and kidney were compared with crude solubilizes of brain containing a similar amount of protein, only the brain samples were immunoreactive with a pattern similar to that of the purified receptor.

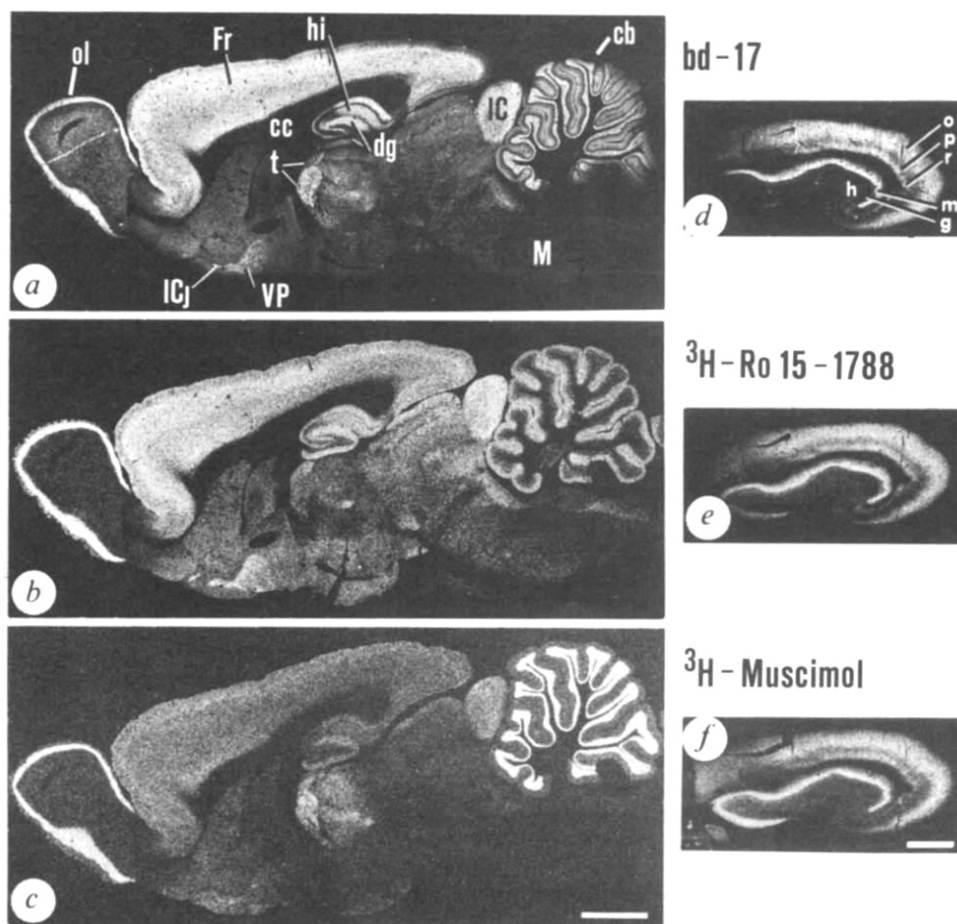
As bd-24 and bd-17 seemed to immunoreact with a GABA_A receptor complex, we attempted to localize it immunohistochemically. An identical pattern of immunological staining was obtained with bd-17 and bd-24 in bovine or human brain sections, whereas in the rat only bd-17 was immunoreactive. (The lack of immunoreactivity of bd-24 in the rat was confirmed by immunoblotting and immunoprecipitation using a purified receptor preparation from rat brain.)

Assuming that the observed immunoreactivity in grey matter represented a GABA_A receptor associated with a benzodiazepine receptor, we expected the staining pattern to be similar to that of benzodiazepine receptors visualized autoradiographically^{16,17} by radioligand binding. Indeed, when semi-adjacent sections were incubated with the benzodiazepine receptor ligand ^3H -Ro 15-1788, the regional distribution and intensity of binding were strikingly similar to that of the immune reaction (Fig. 3), indicating that most benzodiazepine receptors are part of GABA_A receptors. This was shown in rat brain, spinal cord and retina as well as in bovine and postmortem human brain (Fig. 3). Areas which lacked benzodiazepine receptor sites also lacked immunoreactivity, for example, brain white matter, pineal gland and pituitary.

In the granular layer of the rat cerebellum and the ventrolateral thalamus (Fig. 3a), the intensity of the immune reaction was higher than expected from the density of benzodiazepine binding sites (Fig. 3b), corresponding more accurately to the density of high-affinity GABA binding sites visualized autoradiographically with ^3H -muscimol (Fig. 3c). Thus, in these two regions the receptor complex may occur in a conformational

Fig. 3 Immunohistochemical localization of antigenic sites in rat brain (a, parasagittal section) and in post-mortem human hippocampus (d, coronal section) using bd-17: comparison with the autoradiographic localization of benzodiazepine receptor sites (b, e; ^3H -Ro 15-1788 binding) and high-affinity GABA binding sites (c, f; ^3H -muscimol binding). In these negative images, white areas correspond to high densities of antigenic sites and radioligand binding sites.

Methods. a, d, For the immune staining the tissue was fixed with 2% formaldehyde + 0.1% glutaraldehyde in PBS. 10- μm cryostat sections were incubated, according to the indirect peroxidase/anti-peroxidase (PAP) technique²⁵, in sequence with: normal swine serum, hybridoma supernatant bd-17 (5–10 $\mu\text{g ml}^{-1}$), swine anti-mouse antiserum, PAP complex and finally diaminobenzidine/ H_2O_2 . In adsorption controls where bd-17 was preincubated with purified receptor complex, a nonspecific immunoreaction was observed, unless the receptor material was removed subsequently from the sample by a benzodiazepine receptor affinity resin⁴. Benzodiazepine receptor sites and high-affinity GABA binding sites were radiolabelled *in vitro* with 1 nM ^3H -Ro 15-1788 (b, e) and 5 nM ^3H -muscimol (c, f) respectively as described previously^{16,20}. In the presence of 1 μM clonazepam and 10 μM GABA, binding of the respective radioligands was virtually absent. Abbreviations: cb, cerebellum; cc, corpus callosum; Fr, frontal cerebral cortex; dg, dentate gyrus; g, stratum granulosum of the dentate gyrus; h, hilus; hi, hippocampus; ICj, islands of Calleja; IC, inferior colliculus; M, medulla; m, stratum moleculare of the dentate gyrus; o, stratum oriens of the hippocampus; ol, olfactory bulb; p, stratum pyramidale of the hippocampus; r, stratum radiatum of the hippocampus; t, thalamus; VP, ventral pallidum. Scale bars, 2 mm.



state in which high-affinity GABA sites predominate and benzodiazepine binding sites are occluded. Alternatively, these areas may contain GABA_A receptors not linked to benzodiazepine receptors but associated with Cl^- channels. The presumptive Cl^- channel ligand ^{35}S -tert-butylbicyclophosphorothionate¹⁸, tested autoradiographically in rat cerebellum¹⁹, showed the same pattern of distribution and density as the immune reaction in that brain region. If bd-17 and bd-24 cross-react with a GABA_A receptor not linked to a benzodiazepine receptor, a structural homology between two types of GABA_A receptors is suggested. The immunohistochemical staining does not seem to result from a nonspecific immune reaction, as preincubation of either antibody with the purified receptor fraction reduced the intensity of the immune reaction in a concentration-dependent fashion in all parts of the brain. Furthermore, controls with antibody-free medium or with an irrelevant antibody lacked immunoreactivity.

As benzodiazepines are used widely to treat anxiety states, sleep disorders, epileptic convulsions and muscle spasms, dysfunctions of the receptor complex may be involved in the aetiology of some of these disorders. Previous attempts to localize the receptor complex were restricted to the autoradiographical enhancement of radioligand binding to benzodiazepine receptors in micromolar concentrations of GABA²⁰, a technique of limited value for studies of postmortem human brain, as such receptor sites can be occupied through previous treatment with benzodiazepines. Furthermore, unknown amounts of endogenous GABA remaining in the tissue section could interfere with the enhancement of benzodiazepine receptor binding. In contrast, the immune reaction of bd-17 and bd-24 with the purified receptor remained unaltered in the presence

of either GABA (5 mM) or diazepam (20 μM), as tested in a solid-phase antibody assay. Thus, an immunohistochemical investigation of postmortem tissue material can be performed irrespective of the presence of benzodiazepines or GABA and the structure containing the functionally relevant low-affinity GABA site can be visualized unequivocally with monoclonal antibodies. Furthermore, the resolution of the immunohistochemical localization of the receptor complex described here is superior to that of benzodiazepine receptor autoradiography, as single immunoreactive cells with their processes can be visualized (J.G.R., in preparation). Thus, bd-17 and bd-24 may elucidate possible pathological alterations of the GABA_A receptor/benzodiazepine receptor complex in postmortem human brain.

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Action potential mutations stop a biological clock in *Drosophila*

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The *Drosophila melanogaster* male produces a species-specific courtship song by wing vibration. The most conspicuous feature of the song is a series of pulses with a 30–40-ms interpulse interval (IPI)^{1,2} which oscillate in wild-type males with a period of 50–60 s (ref. 3). This short-term biological rhythm in IPI is influenced by several gene mutations at the *period* (*per*) locus, which alter the normal 24-h free-running period of the circadian clock⁴ and have corresponding effects on the song cycle³. The present study reveals that, under restrictive conditions, temperature-sensitive mutations which affect neuronal membrane excitability seem to stop the biological clock underlying the fruitfly's song rhythm.

We have taken advantage of two temperature-sensitive mutations: *nap^{ts}* (no-action-potential temperature-sensitive) and *para^{ts1}* (paralytic temperature-sensitive). Flies carrying these mutations are immobilized rapidly at temperatures >35 °C and 29 °C, respectively^{5,6}, but this paralysis is reversible, both mutants recovering within seconds when returned to 25 °C. At the higher restrictive temperatures, both mutants fail to generate action potentials⁷. These defects in nerve conduction may be caused by abnormalities associated with regenerative sodium channels. Our experimental design allowed individual *nap^{ts}* and *para^{ts1}* males to begin courtship singing to normal females at 25 °C, then heat pulses were applied for 30 s to 'switch off' the male's nervous system. On recovery at 25 °C each male was permitted to continue his courtship. Pre- and post-treatment songs were then analysed to determine whether any phase shifts in the song cycle had occurred.

To control for the effects of the 30-s paralysis induced by failure of nerve conduction in the mutants, normal and mutant males were incapacitated for the same period of 30 s with short 7-s pulses of low temperature or CO₂ (see Table 1 and Fig. 1 legend). These control tests almost certainly do not obliterate membrane excitability except perhaps for a brief period within the 7-s pulse (see, for example, refs 8–11). In the analogous

Table 1 Induced phase shifts of the courtship song rhythm in *Drosophila* males

Genotype	Treatment	n	Mean phase shift (s) ± s.e.m. (advances = +, delays = -)	Range (s)
Experimental				
Wild type	30-s heat pulse (42 °C)	5	+1.5 ± 1.8	-4.9 to +5.0
<i>nap^{ts}</i>	30-s heat pulse (42 °C)	7	-31.2 ± 1.8	-38.2 to -26.1
<i>nap^{ts}cn</i>	30-s heat pulse (37 °C)	8	-28.9 ± 2.5	-39.1 to -21.3
<i>para^{ts1}</i>	30-s heat pulse (37 °C)	8	-31.0 ± 3.1	-39.0 to -16.1
<i>nap^{ts}cn</i>	15-s heat pulse (37 °C)	6	-16.2 ± 3.2	-25.2 to -2.0
<i>para^{ts1}</i>	15-s heat pulse (37 °C)	7	-13.0 ± 2.8	-23.8 to -3.9
<i>nap^{ts}cn</i>	45-s heat pulse (37 °C)	5	-48.4 ± 4.6	-59.3 to -36.3
<i>para^{ts1}</i>	45-s heat pulse (37 °C)	4	-47.5 ± 2.8	-53.9 to -40.8
Controls				
Wild type	Removed from female only	6	+1.0 ± 2.6	-6.7 to +9.9
<i>nap^{ts}cn</i>	Removed from female only	6	+0.5 ± 2.5	-9.9 to +7.0
<i>para^{ts1}</i>	Removed from female only	5	+1.3 ± 3.2	-8.0 to +8.0
Wild type	7-s cold pulse (0 °C)	4	+0.2 ± 2.4	-4.0 to +5.0
<i>nap^{ts}</i>	7-s cold pulse (0 °C)	4	-4.0 ± 1.3	-7.0 to -0.7
Wild type	7-s CO ₂ pulse	5	-2.3 ± 1.2	-4.7 to +5.7
<i>nap^{ts}</i>	7-s CO ₂ pulse	5	-1.8 ± 2.2	-7.1 to +5.3

Two strains carrying the chromosome 2 mutation *nap^{ts}* were used: one of these had been maintained at the California Institute of Technology at 25 °C and these flies were quickly immobilized at 42 °C. A second line, carrying the eye colour mutation *cinnabar* (*cn*) had been maintained at 18 °C by Dr Barry Ganetzky at the University of Wisconsin. The sex-linked *para^{ts1}* mutation has also been maintained at 18 °C in Wisconsin, and both *nap^{ts}cn* and *para^{ts1}* flies showed nearly instantaneous paralysis at 37 °C. The males were placed in a preheated glass tube at either 37 °C or 42 °C and maintained at this temperature for 15, 30 or 45 s, as indicated. *nap^{ts}* and *para^{ts1}* males became immobilized within 1–2 s at these temperatures and remained motionless for the duration of the heat pulse. The mutants were then removed from the tubes and replaced in the mating chamber at 25 °C where they regained full mobility within 2–5 s. The wild-type males did not 'pass out' during similar high-temperature treatment. Females were then re-introduced to the males. In the no-treatment controls, the male was simply removed from the female for 60 s and then returned. In this test the total time for which the male was removed from the female (including 20–25-s handling time) was 80–85 s, which corresponds to approximately 1½ cycles of the normal 55-s song rhythm. Thus, if this treatment arrested the clock, the song rhythm would be approximately half a cycle out of phase when the male resumed his courtship. In a further control test, the male was immobilized in a chilled glass tube at 0 °C. All flies were immediately disabled at this temperature and usually remained in torpor for 25–35 s if the cold pulse was maintained for no longer than 7 s. Any male remaining motionless for less than 25 or more than 35 s was discarded. In a final control treatment, the courting male was removed from the female and placed in a small mating cell, into which CO₂ was blown for 7 s. Only those males that were anaesthetized and remained motionless for between 25 and 35 s were subsequently returned to the female. After each treatment males were allowed to continue their courtship for 5–8 min. Flies that had not resumed courtship 5 min after the end of the pulse were eliminated from further analysis. Males which did not sing vigorously either before or after the treatment were similarly discarded. Between 10 and 15 males were observed initially in each experiment but many did not court at all, either before or after the treatment, or sang so weakly that further analysis was not warranted. A vigorous courtship was operationally defined as one in which an average of 25 individual IPIs could be measured per 10 s of courtship time. Phase shifts were computed as described in Fig. 1 legend. Of the 170 songs analysed above (85 pre- and 85 post-treatment songs), 113 were significantly rhythmic beyond the 1% level (see ref. 3), 51 were significantly rhythmic beyond the 5% level and 6 just missed significance at 5%. In addition, 14 males sang vigorously but were arrhythmic in either their pre- or post-treatment songs. n, The number of males in each experimental group whose courtship song was analysed. Males were tested once only.

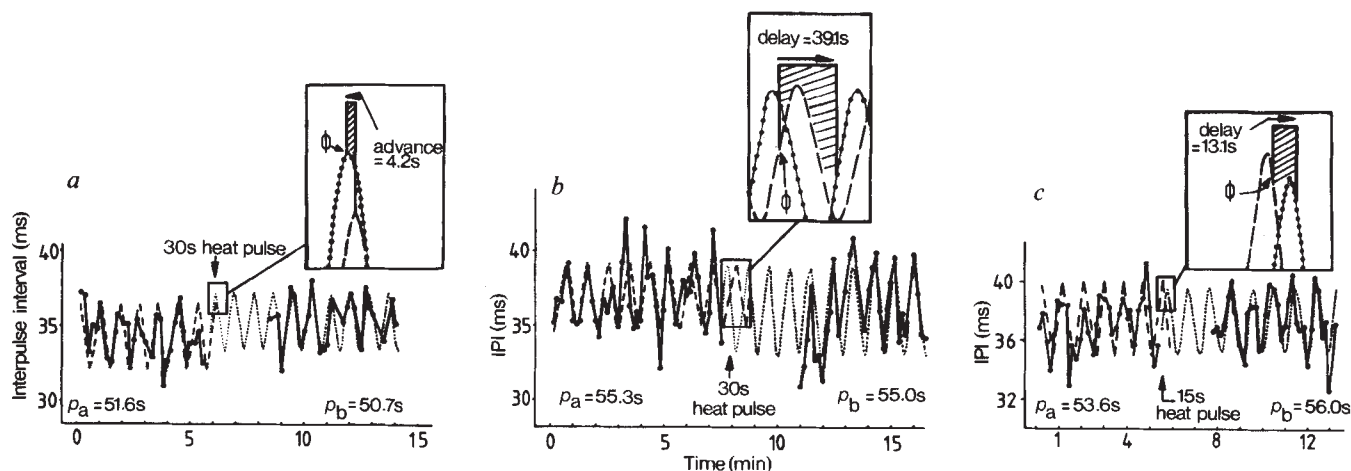


Fig. 1 Induced phase shifts of the song rhythms of *nap^{ts/cn}* and wild-type *Drosophila* males. Each point represents the mean IPI per 10 s of courtship time (values of s.e.m. have been omitted for clarity, but 80% of all the s.e.m. values fall in the range 0.4–1.2 ms). Tape recordings of the courtship songs of 5-day-old males to 2-day-old virgin, attached-X chromosome females were made from a small mating cell using a ribbon microphone and were then input to an oscillograph to provide a visual record of the pulse trains³. IPIs were measured peak-to-peak with a ruler using minimum and maximum cut-off points of 15 and 65 ms, respectively. Song rhythms were obtained by calculating the mean IPI for successive 10-s time frames and placing these means into a computer together with approximate initial values for the parameters of a sine function. These parameters included the phase, period and amplitude of the rhythm, and the value around which the cycle oscillated. An iterative programme was run twice for each male's song, once to obtain the best-fitting sine wave for the pretreatment IPI means (dashed line) and then again for the post-treatment means (dotted line). The computer analysis was performed only after both sets of data (that is, pre- and post-treatment IPI means) had been calculated for each male. The goodness-of-fit of each set of data to its corresponding sine wave was examined using an *F*-test, yielding a measure of how 'sinusoidal' or 'rhythmic' the data were. Estimates of the various sine-wave parameters were used to calculate the phase shift between the pre- and post-treatment song rhythms. Because of the frequent small differences in the periods of the pre- and post-treatment songs (p_a and p_b , respectively), the phase shift between the two curves was calculated at the moment the treatment pulse was applied. **a**, Song profile for a wild-type male subjected to a heat pulse (42 °C) for 30 s. The pretreatment song period, p_a , is 51.6 s; p_b = 50.7 s. The heat pulse was administered 370 s after the beginning of the courtship song, and when the pre-treatment song rhythm is extrapolated to this point, the phase at which the heat stimulus was applied, Φ , is 1.57 radians (this value is the peak of the cycle). Extrapolating the post-treatment song rhythm back to this point, the time coordinate which represents a phase of 1.57 radians for the post-treatment curve is 365.8 s. Thus the two peaks are 370–365.8 s, or +4.2 s, out of phase. This represents an advance of 4.2 s of the post-treatment song rhythm on that of the pretreatment. The shaded area in the inset represents this phase advance. **b**, The song rhythm of a *nap^{ts/cn}* male undergoing a paralysing 30-s heat pulse (37 °C) administered 470 s after the male initiated courtship singing. This time point corresponds to a phase of 5.95 radians on the pretreatment curve. The corresponding coordinates for Φ = 5.95 radians on the post-treatment curve fall at 454.1 s and then at 509.1 s on the following cycle, yielding phase shifts of either 470–454.1 = +15.9 s (advance), or 470–509.1 = –39.1 s (delay). We assumed initially that this phase shift was a delay (see text). The shaded area in the inset again represents the phase delay. **c**, The song produced by a *nap^{ts/cn}* male subjected to a 15-s paralysing heat pulse (37 °C), at 340 s. This time point represents Φ = 1.92 radians (again like **a**, the heat pulse is initiated near the peak of the cycle) on the pretreatment curve. The corresponding time coordinate represented by this phase angle falls at 353.1 s on the post-treatment curve. The phase shift is therefore 340–353.1 = –13.1 s (delay).

mutant cases, action potentials are blocked for the duration of the paralysis. The no-treatment control represents a systematic test of our earlier observation that when a male resumes singing after previously interrupting courtship, the song cycles remain in-phase with those established earlier³.

Table 1 summarizes the phase shifts induced by the various treatments and Fig. 1 illustrates some individual results. Mutant and wild-type males which received no treatment, except for a 60-s absence from the female, exhibited mean phase shifts of <2 s. Wild-type males subjected to 42 °C pulses also showed small phase shifts. In the case of the mutants undergoing 30-s high-temperature pulses, we assumed initially that all phase shifts were delays; in these three groups the song rhythms were phase-delayed by ~30 s. In contrast, mutant and wild-type flies immobilized for between 25 and 35 s with 'cold' or CO₂ pulses showed small (<5-s) phase shifts. The small phase shifts in the controls can be classified clearly as advances or delays. The large (~30 s) phase shifts in the heat-pulsed mutants, however, are more difficult to categorize because 'delays' greater than half the song period can also be defined as advances. In Fig. 1b, which illustrates the song profile of a *nap^{ts/cn}* male subjected to a 30-s heat pulse, the large 39.1-s delay could also be interpreted as an advance of 15.9 s. We therefore applied 15-s heat pulses to courting *nap^{ts/cn}* and *para^{ts}* males in the same conditions as before; we observed that all phase shifts could be unambiguously categorized as delays (that is, <180°), with the average phase shifts for the two mutant groups being ~15 s (see Table 1). Thus, we believe that the previous 30-s heat treatments similarly caused delays in the mutants. To confirm this interpretation of

the data, we also applied 45-s heat pulses to courting *nap^{ts/cn}* and *para^{ts}* males. Our results supported the delay hypothesis in that large delays of ~45 s were observed in both groups (see Table 1). Thus, we conclude that 'shutting down' the nervous system in these mutants with heat pulses leads to a stoppage of the song clock proportional to the duration of the heat stimulus.

This is a novel result which does not have a parallel in the circadian literature. Circadian rhythms often reveal phase response characteristics when pulses of heat or cold are applied to a 24-h rhythm at various points in its cycle^{12–14}. In the *nap^{ts}* and *para^{ts}* mutants, however, the song clock simply seems to 'stop' at restrictive temperatures and to start again when the fly is returned to the permissive temperature. Unfortunately, because of the small numbers of flies tested, it was difficult to determine whether there were any phase-response patterns in the songs of wild-type flies undergoing temperature pulses. Larger scale experiments using wider ranges of hot and cold pulses may be required if any phase-response curves are to be obtained for the song rhythm.

The *nap^{ts}* and *para^{ts}* mutations which we have used to block neuronal conductance act synergistically in that double mutants do not survive at any temperature⁷, suggesting that the mutations share a common membrane excitability function. *nap^{ts}* seems to alter the density of sodium channels as deduced by tetrodotoxin (TTX) and saxitoxin binding studies^{15–17}, and wild-type flies given a sub-lethal dose of TTX, which blocks and therefore reduces the number of membrane-bound sodium channels, show temperature-sensitive paralysis similar to that of *nap^{ts}* flies¹⁵. Perhaps, therefore, the two mutations interrupt the

Drosophila song clock because of the direct⁷ or indirect¹⁵ effects of these genetic variants on the structure and function of sodium channels in neural membranes. Pharmacological manipulations of ionic fluxes change the phasing of a circadian oscillator localized in the *Aplysia* eye^{18,19} but TTX does not phase-shift this neural rhythm²⁰. In view of the latter result, it would be useful to examine whether the *nap^{ts}* and *para^{ts}* mutations lead to a temperature-sensitive arrest of the circadian clock in flies. There is no reason to assume that every perturbation which affects the song clock will similarly affect the circadian clock, even though both rhythms share a common genetic mechanism via the effects of the *per* mutations³. For example, constant light disrupts the circadian clock, but not the song oscillation³. Therefore, the *nap^{ts}* and *para^{ts}* mutations can be used to probe the differences as well as the similarities between short-cycling and circadian rhythms. The striking stoppage of the fly's song clock by block of neuronal conductance with the *nap^{ts}* or the *para^{ts}* mutations indicates that this short-term rhythm is regulated by a neural oscillator. Genetic procedures²¹ are available for localizing these presumptive neural 'foci', by constructing genetic mosaics which express *nap^{ts}* or *para^{ts}* in their mutant

tissues. Such studies may reveal whether these mutations lead to an interruption of the song cycle by affecting a small localized region within a ganglion or whether several sites (each perhaps containing a separate oscillator) must be simultaneously mutant in order for such arrests to be observed. In the latter case, communication may be blocked between oscillators which need to be coupled to regulate the phase of the rhythm; these foci may be the same or quite different from the sites of action of the *per* gene. Mosaic experiments on circadian behaviour have shown that the anatomical focus for the short-period *per^s* variant is localized in the fly's head²². Further work on the effects of the *nap^{ts}*, *para^{ts}* and *per* mutations should help to elucidate the complex relationship between the mechanisms controlling short-term and circadian rhythms in *Drosophila*.

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Comparative chromosome mapping of a conserved homoeo box region in mouse and human

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Specific genes are assumed to regulate pattern formation in the mammalian embryo, but as yet none has been identified unequivocally. It is possible that such genes in mammals may be identified by virtue of a conserved coding sequence, because many of the *Drosophila melanogaster* homoeotic and segmentation genes, which have crucial roles in the regulation of segmental pattern formation during embryonic development^{1,2}, contain a 180-base pair (bp) DNA sequence, the homoeo box³⁻⁵, and that sequences homologous to the *Drosophila* homoeo box are also present in 6-10 copies in higher animals, including mammals⁴⁻⁸. Although the assumption that the homoeo box identifies genes responsible for pattern formation in mammals remains to be validated, it is a particularly attractive hypothesis given the strong conservation of homoeo boxes over vast evolutionary distances. Here we report the localization of a human homoeo box region⁷, previously cloned and shown to contain two homoeo boxes within a sequence of 5-kilobases (kb), to the long arm of chromosome 17. We show that two single-copy homoeo box-flanking probes derived from this region strongly hybridize to single-copy restriction fragments in mouse genomic DNA and that these conserved homoeo box-flanking sequences map to mouse chromosome 11. This may be significant as several genes that map to chromosome 17 in human also map to chromo-

some 11 in the mouse, implying that a segment of mouse chromosome 11 is homologous to a region of human chromosome 17 (ref. 9). Taken together, these data suggest that the homoeo box region detected with our probes is highly conserved in human and mouse.

To determine the chromosomal location of the human homoeo box region, two single-copy probes were constructed. Figure 1 shows a restriction map of the cloned region of the human genome, containing two linked homoeo boxes⁷ designated *Hu-1* and *Hu-2*, and illustrates the probes derived from it. One probe, H.1, is a 1.7 kb *Hind*III fragment containing the *Hu-1* homoeo box; the other probe, LF.2, is a 1.2-kb *Hind*III/*Eco*RI fragment containing sequences flanking the *Hu-2* homoeo box on the side opposite *Hu-1* or on the left side of *Hu-2* as the map is presented. Southern blot analysis has shown that the H.1 probe detects a single restriction enzyme fragment in human DNA when

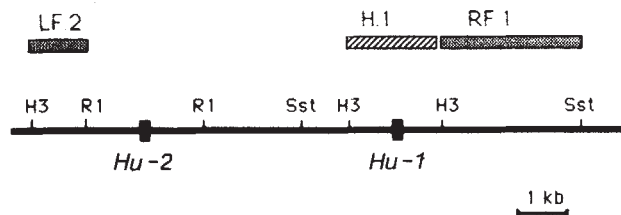


Fig. 1 Restriction map of the human homoeo box region studied. The probes used for the mapping studies are indicated. The restriction map was derived from two clones, λ Hu1 and λ Hu2, described elsewhere⁷. The horizontal line represents the human DNA sequence and the two small solid boxes represent homoeo boxes. The exact position of each of the homoeo boxes between the two restriction sites indicated has not been determined. The stippled and cross-hatched boxes above the restriction map represent the probes derived from this region. LF.2, or left flanking sequence to *Hu-2*, is a 1.2-kb *Eco*RI/*Hind*III fragment; H.1 is a 1.7-kb *Hind*III fragment containing the *Hu-1* homoeo box; and RF.1, or right flanking sequence to *Hu-1*, is a 2.4-kb *Hind*III-*Sst*I fragment. These fragments were subcloned into the vector SP65 and the recombinant plasmids (pLF.2, pH.1 and pRF.1) were used as probes. R1, *Eco*RI; H3, *Hind*III; Sst, *Sst*I.

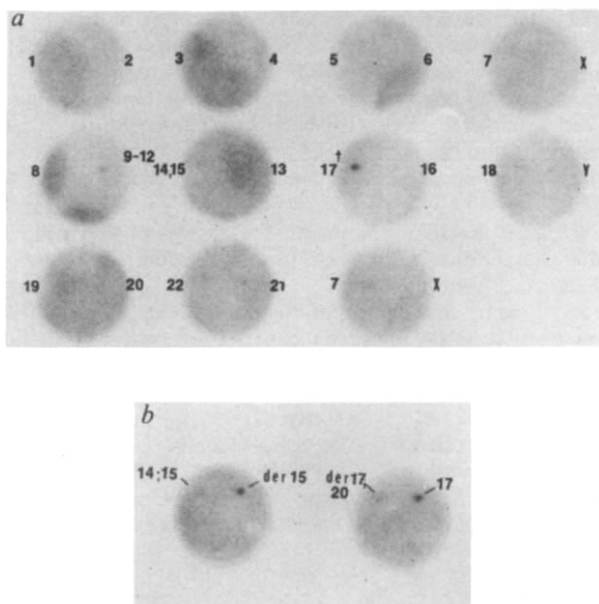


Fig. 2 Assignment of the human homoeo box region to the long arm of chromosome 17. *a*, Autoradiograph of a panel of spot blot filters containing flow-sorted human chromosomes hybridized to the LF.2 probe. *b*, Autoradiograph of spot blot filters containing flow-sorted chromosomes from a cell line containing a 15;17 reciprocal translocation hybridized to the LF.2 probe.

Methods. Chromosomes (30,000) of each type were either sorted directly onto spots on nitrocellulose filters, denatured and neutralized as previously described^{10,11} or sorted directly into Leif buckets and identified by quinacrine-banding analysis. The filters were prehybridized overnight at 42 °C in 5×SSC, 5×Denhardt's solution, 250 µg ml⁻¹ sonicated boiled salmon sperm DNA, 50 mM NaPO₄, pH 7, 0.1% SDS and 50% deionized formamide and hybridized for 48 h at 42 °C in the same solution containing nick-translated ³²P-labelled pLF.2 (3×10⁶ c.p.m. ml⁻¹). After hybridization, the filters were washed three times for 5 min each at room temperature and twice at 50 °C for 30 min each in 2×SSC and 0.1% SDS, followed by one wash in 0.2×SSC for 30 min at 65 °C.

hybridized and washed in stringent conditions⁷. LF.2 also hybridizes to a single-copy sequence in human genomic DNA (see below).

To determine which human chromosome contains the homoeo box region, we used dual-laser chromosome sorting and spot-blot analyses^{10,11}. Two panels of human chromosomes sorted directly onto nitrocellulose filters (spot blots) were hybridized with the H.1 and LF.2 probes and washed at high stringency (see Fig. 2 legend). Figure 2*a* shows an autoradiograph of a spot blot hybridized to LF.2. A specific signal is detected only in the fraction containing chromosome 17; the weak signal detected in the fraction containing a pool of chromosomes 9–12 is observed with most probes and is presumably nonspecific background which is more prominent in this dot owing to the presence of more DNA than in dots containing only one chromosome. As expected, hybridization of a similar panel of chromosomes to H.1 gives an identical result (data not shown).

In order to sub-localize the homoeo box-related sequences on chromosome 17, we sorted chromosomes from human fibroblasts heterozygous for a reciprocal translocation between the long arms of chromosomes 17 and 15 [46 XX, t(15;17) (q22;q11)] (M. LeBeau and J. Rowley, personal communication). The normal chromosome 17 and the derivative 15 [der(15)], which contains the long arm of chromosome 17, are each sorted separately, whereas the derivative 17 [der(17)] which contains the short arm of chromosome 17, is sorted with chromosome 20 (not shown). Chromosomes 14 and 15 are sorted together and were spot-blotted as a negative control. Figure 2*b* shows an autoradiograph of the spot blot from this chromosome sort hybridized to the LF.2 probe. A specific signal is detected

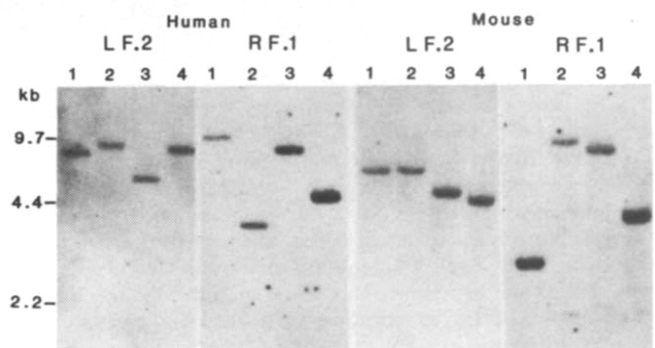


Fig. 3 Hybridization of the homoeo box flanking probes, LF.2 and RF.1, to human and mouse DNAs. The DNAs were restricted by the following enzymes: 1, *Eco*RI; 2, *Bam*HI; 3, *Hind*III; 4, *Sst*I.

Methods. Total cellular DNAs (10 µg) isolated from human and mouse tissues were cleaved with the restriction enzymes indicated. After gel electrophoresis and transfer to nitrocellulose, the baked filters were hybridized to pLF.2 and pRF.1 probes as described in Fig. 2 legend, except that the last wash in 0.2×SSC was at 50 °C and in some cases the filters were prehybridized and hybridized at 64 °C in 6.6×SSC, 10×Denhardt's, 0.1% SDS, 0.1% sodium pyrophosphate.

in the two fractions containing chromosome 17 and [der(15)]. Thus, these experiments localize the human homoeo box region shown in Fig. 1 to the long arm of chromosome 17.

Comparative gene mapping has demonstrated genetic homology between the long arm of human chromosome 17 and mouse chromosome 11, hence if the homoeo box region on human chromosome 17 is conserved in the mouse, it may map to mouse chromosome 11 (ref. 9). To test this hypothesis, we first demonstrated that DNA sequences homologous to the human chromosome 17 homoeo box region are present in mouse genomic DNA. In addition to the *Hu-2*-flanking probe LF.2, we constructed a 2.4-kb *Hu-1*-flanking probe (RF.1) containing sequences adjacent to *Hu-1* on the side opposite *Hu-2* or on the right side of *Hu-1* (see Fig. 1). The Southern blot experiments shown in Fig. 3 demonstrate that the LF.2 and RF.1 probes detect specific restriction fragments in human DNA cut with a variety of enzymes, indicating that each probe recognizes a different single-copy sequence in human DNA. The fragment sizes detected are those predicted from the restriction map of the human clones shown in Fig. 1. To demonstrate homology between these flanking probes and mouse DNA sequences, we hybridized a Southern blot of mouse genomic DNA restricted with a variety of enzymes to LF.2, and RF.1 under moderate stringency (see Fig. 3 legend). Figure 3 shows the results of such an experiment. The *Hu-1*- and *Hu-2*-flanking probes detect specific restriction enzyme fragments with each of the enzymes used.

To determine whether the single-copy mouse DNA sequences homologous to the probes LF.2 and RF.1 map to mouse chromosome 11, we used rat-mouse and Chinese hamster-mouse somatic cell hybrids. RTM9, a rat-mouse somatic cell hybrid clone, was formed by polyethylene glycol-mediated fusion of thymidine kinase-deficient Rat-2 cells with spleen cells from a mouse homozygous for an 11;13 robertsonian translocation chromosome, followed by selection in hypoxanthine-aminopterin-thymidine medium¹². The murine 11;13 translocation chromosome is the only mouse chromosome in this hybrid clone and is present in over 90% of RTM9 metaphases analysed by standard trypsin-Giemsa banding techniques (data not shown). The mouse thymidine kinase gene is present on mouse chromosome 11. Thus, by growing RTM9 in medium containing 5-bromodeoxyuridine, we were able to isolate a subclone, RTM9-Bu, that lacks the mouse 11;13 chromosome but retains the same rat chromosome complement as the parental clone, as determined by karyotype analysis. We also tested a Chinese hamster-

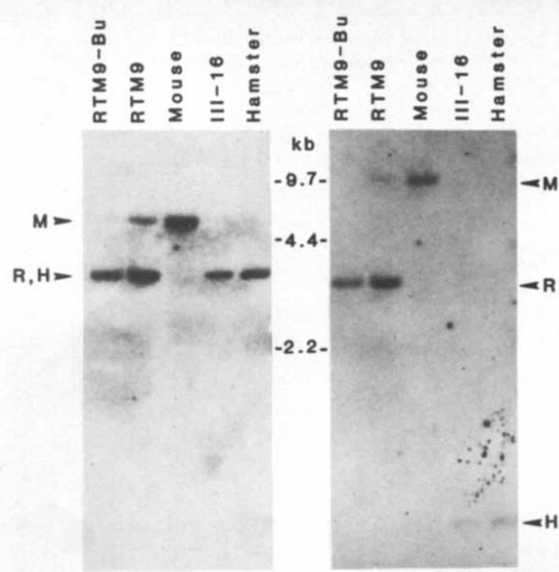


Fig. 4 Detection of mouse-specific LF.2 and RF.1 homologous sequences in DNA from the hybrid cell line RTM9, which contains mouse chromosomes 11 and 13, and demonstration of their absence from the cell hybrid III-16, which contains several mouse chromosomes including 13, but not 11. R, rat; M, mouse; H, hamster.

Methods. Total cellular DNAs were isolated from normal mouse tissues, the rat-mouse somatic cell hybrid RTM9, the back-selected subclone RTM9-Bu, which contains no mouse chromosomes but retains the same complement of chromosomes as the parental clone, the Chinese hamster-mouse hybrid III-16, and the hamster cell line 380-6 that was used to construct III-16. These DNAs were cleaved with *Hind*III, Southern-blotted and hybridized to pLF.2 and pRF.1 probes as described in Fig. 3 legend.

families such as the globin genes, which demonstrate that coding regions are strongly conserved whereas introns and flanking sequences are generally not conserved¹³. Whether the genes found within this human/mouse homoeo box region have an important role in the control of mammalian development remains to be determined. In this context it may be relevant that two mutations that affect the number and form of the vertebrae have been mapped¹⁴ to mouse chromosome 11: *vestigial-tail* is a viable recessive mutation, and *tail-short* is a dominant mutation which is viable in the heterozygous condition, but which causes embryonic death in the peri-implantation period when homozygous. It will be of interest to determine whether either of these mutations is allelic to the mouse cognate of the human homoeo box region that we have assigned to mouse chromosome 11.

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Two homoeo box loci mapped in evolutionarily related mouse and human chromosomes

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The homoeo box is a 180-base pair (bp) DNA sequence conserved in *Drosophila* homoeotic genes, which regulate early development^{1,2}. These DNA sequences are present in open reading frames and have been identified in specific gene transcripts in *Drosophila* and *Xenopus* embryos³⁻⁵; they possess structural features in common with genes encoding some DNA-binding proteins^{6,7}. Homologous homoeo box sequences have been detected in species ranging from insects and annelids to vertebrates^{2,5,8}. The high degree of sequence conservation (70-90%) among different species suggests a strong evolutionary relationship and implies a common role in embryonic development. To test this hypothesis, one approach we have used is to examine the patterns of genetic organization of homoeo box sequences in mouse and human for any similarities; the second approach is to localize the chromosomal map positions of homoeo box sequences in the two species. A similar genomic organization and chromosomal distribution of homoeo box sequences would argue for a conserved function and might shed light on their mechanism of action. Here, we describe experiments which show that two homoeo box loci map, respectively, to evolutionarily related regions on mouse chromosome 11 and human chromosome 17.

mouse somatic cell hybrid, III-16 (ref. 12), containing mouse chromosome 13 as well as several other mouse chromosomes but lacking mouse chromosome 11. Genomic DNA was isolated from normal mouse tissues, RTM9, RTM9-Bu, III-16, and the Chinese hamster parental cell line 380-6 that was used to construct III-16. The murine DNA fragments detected by LF.2 and RF.1 were distinguished from the homologous hamster or rat DNA fragments on the basis of size after *Hind*III restriction enzyme digestion, followed by Southern transfer and hybridization at moderate stringency (see Fig. 3 legend). Figure 4 shows that the mouse-specific DNA fragments that hybridize with the LF.2 and RF.1 probes are present in RTM9 DNA, but not in DNA from RTM9-Bu or III-16. Results of a similar study using the H.1 probe were difficult to interpret because, in the moderate hybridization conditions used, this homoeo box-containing probe detects 6-8 DNA fragments in mouse, hamster and rat DNA. Taken together, these results demonstrate that the single-copy DNA sequences homologous to those in the human chromosome 17 homoeo box region are linked in the mouse, as they are in human, and map to chromosome 11.

The extent to which the homoeo box region is conserved between human and mouse will remain unknown until the homologous mouse *Hu-1*- and *Hu-2*-flanking sequences have been cloned and sequenced. The availability of the murine genomic clones should also make it possible to determine the number of homoeo boxes in this region. By analogy with human, we expect that this region of mouse chromosome 11 contains at least two homoeo boxes. This suggests that there may be clustering of homoeo boxes in mammals, as is the case in *Drosophila*. However, not all homoeo box-containing sequences map to mouse chromosome 11—at least one homoeo box sequence maps to mouse chromosome 6 (ref. 8), indicating that there is some dispersion of homoeo boxes in the mouse genome.

The fact that the human chromosome 17 homoeo box region is conserved in mouse suggests that this region is indeed part of a coding sequence in the mammalian genome. This conclusion is based on data from comparative sequence studies of gene

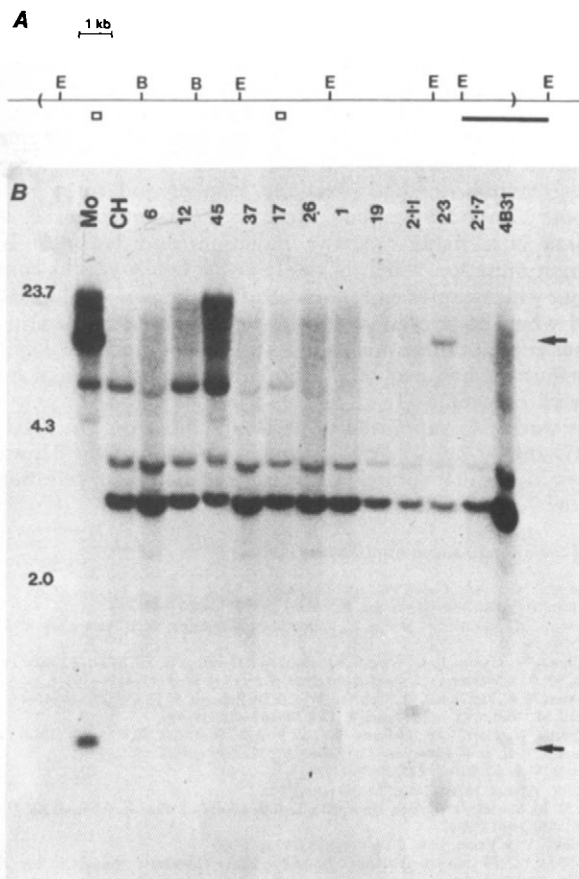


Fig. 1 **A**, Restriction map of the *Hox-2* recombinant phage insert. Boxes beneath the line indicate the two restriction fragments containing sequences homologous to the *Drosophila* homoeo box. The hybridization probe used for chromosomal mapping is underlined by the solid bar. The brackets indicate vector/insert cloning sites. **B**, *Bam*HI; *E*, *Eco*RI. **B**, Southern blot hybridization of mouse, hamster and mouse x hamster hybrid cell DNAs with the *Hox-2* DNA probe indicated in **A**. The *Hox-2* probe was nick-translated with 32 P-dCTP to a specific activity of 10^8 c.p.m. μg^{-1} and hybridized as reported previously⁹. The murine chromosome complement of the hybrid cell lines was determined by karyotyping and/or analysis of isozyme and DNA markers.

A recombinant phage clone containing murine DNA homologous to *Drosophila* homoeo box sequences was isolated from a mouse genomic library as reported previously⁹. Restriction site mapping of the murine DNA insert described here showed that this clone was not derived from the homoeo box locus (*Hox-1*) present on mouse chromosome 6 (ref. 9), hence we call this new region *Hox-2*. Southern blot hybridization of restriction enzyme-digested *Hox-2* DNA with a *Drosophila* homoeo box probe revealed two distinct regions of homoeo box sequence homology, 5 kilobases (kb) apart. This structural organization was similar to that reported for two linked homoeo boxes in the human genome (*Hu-1* and *Hu-2*)⁸, and suggests that the *Hox-2* locus in the mouse and the region spanning the *Hu-1* and *Hu-2* probes in man may represent homologous genomic domains. As an initial step in the comparison of these homoeo box regions in mouse and human, we have mapped these loci to determine whether they are localized in evolutionarily related chromosomal regions.

To map the cloned mouse sequences, 20 mouse x hamster hybrid cell DNAs were probed with a 2.7-kb *Eco*RI fragment flanking the homoeo box regions of the *Hox-2* recombinant phage insert (Fig. 1A). The murine DNA contained in this fragment hybridizes with 0.9-kb and 10-kb *Hind*III fragments in a mouse genomic Southern blot. A strongly cross-hybridizing band of 3 kb is present in the hamster genome. In addition,

Table 1 Segregation of *Hox-2* in mouse-hamster hybrids (presence of chromosome/blot hybridization)

Mouse chromosome	Concordant			Discordant		
	+/+	-/-	Total	+/-	-/+	Total
1	1	9	10	9	1	10
2	1	9	10	9	0	9
3	1	10	11	9	0	9
4	1	9	10	11	0	11
5	1	12	13	6	1	7
6	1	7	8	12	0	12
7	1	5	6	14	0	14
8	1	12	13	7	0	7
9	1	6	7	12	1	13
10	1	9	10	10	0	10
11	1	18	19	0	0	0
12	1	3	4	16	0	16
13	0	3	3	4	1	5
14	1	16	17	2	1	3
15	1	3	4	5	0	5
16	0	4	4	4	1	5
17	1	7	8	12	0	12
18	1	13	14	2	0	2
19	1	7	8	10	0	10
X	1	2	3	12	0	12

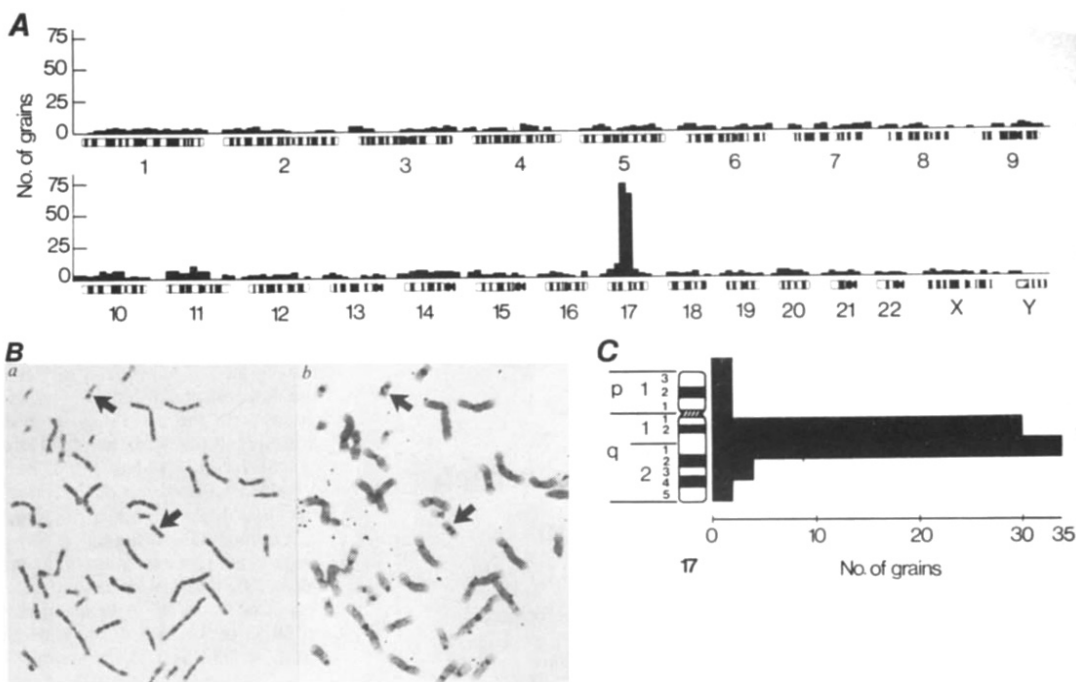
Summary of the blot hybridization results for 21 mouse x hamster hybrid cell line DNAs probed with the *Hox-2* DNA fragment (see Fig. 1A). The presence or absence of mouse chromosome 11 is concordant with the presence or absence of the *Hox-2* diagnostic mouse bands.

other more weakly hybridizing bands are seen in both the mouse and hamster genomes (Fig. 1B). Scoring the hybrid cell lines for the *Hox-2* mouse-specific fragments assigns this genomic locus to mouse chromosome 11. Table 1 summarizes the data, showing no discordancies either for the presence or absence of chromosome 11 in the hybrids or for the presence or absence of the diagnostic murine restriction fragments.

The human homoeo box sequences, *Hu-1* and *Hu-2*, were regionally mapped by *in situ* hybridization to human metaphase chromosomes^{10,11}. The chromosomes were identified by G-banding before hybridization. After hybridization and autoradiography, the distribution of silver grains over 73 previously photographed chromosome spreads was plotted on a histogram (Fig. 2A) in which a standard idiogram of the haploid human karyotype was divided into 238 units scaled to the average size of a silver grain (0.35 μm). Of 712 grains assigned to chromosomes, 140 (20%) were specifically localized to 17q11-q22, which represents 0.8% of the haploid genome length. Statistical evaluation (by Poisson distribution) of the number of grains per unit chromosome length confirmed a highly significant concentration of label within this region ($P < 0.001$). Figure 2B shows a representative chromosome spread hybridized with the *Hu-1* and *Hu-2* probes. Analysis of a further 24 labelled chromosomes 17 from 30 metaphase spreads (Fig. 2C) illustrates the localization of the *Hu-1* and *Hu-2* homoeo box probes to 17q11-q22.

Comparative mapping between mouse and human has revealed conservation of syntenic linkage associations for several chromosomes¹²⁻¹⁴. Here we present data indicating that two mouse and human homoeo box loci map to evolutionarily related chromosome regions in these species. The human *Hu-1* and *Hu-2* clones have been regionally localized to 17q11-q22; also present at this site are the pro- α 1(I) collagen gene^{15,16}; the thymidine kinase (TK) and galactokinase (GALK) genes, which have been shown to be tightly linked (within 1.2 centimorgans)¹⁷⁻¹⁹; a cluster of genes for growth-regulating hormones, including growth hormone and CSH^{20,21}; and the cellular homologue of the *erb-A1* oncogene, which is believed to enhance the transforming potential of leukaemogenic cells^{22,23}. The mouse *Hox-2* locus has been assigned to chromosome 11; the murine TK and GALK genes have previously been mapped 71 centimorgans from the centromere of this chromosome^{24,25}. As

Fig. 2 Regional localization of the human *Hu-1* and *Hu-2* homoeo box sequences. **A**, Distribution of autoradiographic silver grains over 73 chromosome spreads after *in situ* hybridization with 125 I-labelled *Hu-1* and *Hu-2*. The data were plotted on a histogram representing the haploid human karyotype. If 712 grains appearing on chromosomes, 140 (20%) were localized within the region 17q11-q22. **B**, G-banded chromosome spread (JS cell line) before (**a**) and after (**b**) hybridization. The arrows indicate *Hu-1* and *Hu-2* signal on chromosome 17. **C**, Distribution of silver grains on labelled chromosomes 17 from 30 metaphase spreads, showing localization of the *Hu-1* and *Hu-2* homoeo box sequences to 17q11-q22.



Methods. Human metaphase chromosome spreads were prepared by standard techniques from cultures of karyotypically normal Epstein-Barr virus-transformed lymphoblastoid cell lines (JS, DB and PM). *Hu-1* and *Hu-2*, containing 1.7- and 2.1-kb human DNA inserts, respectively⁸, were mixed 1:1 and nick-translated with 125 I-dCTP (2,000 Ci mmol⁻¹; Amersham) to a specific activity of 6.4×10^8 d.p.m. μ g⁻¹ (ref. 31). Unincorporated nucleoside triphosphates were removed by CF11 cellulose chromatography³². Chromosome preparations were hybridized with 125 I-labelled *Hu-1* and *Hu-2* probe mix (0.15 μ g ml⁻¹) at 37 °C for 16 h and autoradiographed for 7–21 days as described previously^{10,11}.

linkage of the genes for TK and GALK has been conserved in both species, the mouse *Hox-2* locus probably maps close to these genes. These data also suggest that the *Hox-2* locus in the mouse is homologous to the genomic domain containing the human homoeo box regions *Hu-1* and *Hu-2*. It will be interesting to determine whether the murine *erb-1* gene which has been assigned to mouse chromosome 11, maps within this linkage group also.

Genetic mapping studies can suggest or eliminate possible allelism of the homoeo box loci with genomic loci known to affect mammalian development. In the mouse, a gene not ruled out by such comparison is *tail-short* which maps 65 centimorgans from the centromere of mouse chromosome 11 (ref. 26). Homozygotes for *tail-short* do not survive beyond day 5½ of gestation; heterozygotes have skeletal abnormalities which include vertebral fusions, bilateral asymmetry of limb length, triphalangy and an additional pair of ribs. Fine-structure mapping of the *Hox-2* locus using restriction fragment length polymorphisms and recombinant inbred strains of mice could indicate a coincident map position and justify investigation at the molecular level of the relation between the *Hox-2* locus and the *tail-short* mutation. A similar rationale has been proposed to test for allelism between the *Hox-1* locus and hypodactyly, another mutation affecting morphogenesis, which both map to the same region on chromosome 6 (ref. 9). While the function of the mouse homoeo box loci remains speculative, the recent observation that they are transcriptionally active only at specific stages of mouse embryogenesis (C.P.H., Awgulewitsch and F.H.R., in preparation) indicates that their expression is subject to strict temporal regulation, as expected for a gene which in turn modulates morphogenetic patterns. Dysmorphic syndromes in humans include dyssegmental dysplasias²⁷ and anisopondylic compotomicmicromelic dwarfism (dyssegmental dwarfism)^{28,29}, which are neonatally lethal genetic lesions characterized by vertebral segmentation defects. Recent evidence³⁰ suggests an association between dyssegmental dwarfism and defects in the metabolism of $\alpha 1$ collagen, the gene for which maps to the same region of chromosome 17 as the homoeo box loci reported

here^{15,16}. As these cases represent rare occurrences in the general population, there is insufficient genetic data to indicate involvement of homoeo box loci at present. To obtain a better understanding of the function of homoeo box regions during development, studies are under way to compare the temporal and spatial expression of homoeo box sequences during embryogenesis in mouse and man, by blot hybridization to mRNA purified from embryos obtained at progressive stages of gestation and by *in situ* hybridization to endogenous mRNAs in embryo tissue sections. If these genetic loci behave as predicted by analogy to the *Drosophila* homoeotic genes, such data will provide insights into the molecular events controlling mammalian development.

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A *Drosophila* genomic sequence with homology to human epidermal growth factor receptor

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Vertebrate genomes contain an extensive family of genes possessing varying degrees of homology to the *v-src* oncogene. Most *src*-related proteins identified to date are intracellular and membrane-associated, although some are transmembrane proteins and function as receptors for peptide growth factors¹⁻⁶. Three *Drosophila* gene sequences related to the *v-src* gene have been identified, each exhibiting a high degree of homology to one or more of the *src*-family members encoding an intracellular protein⁷⁻⁹. We have isolated a panel of cloned *Drosophila* sequences exhibiting weak *v-src* hybridization and were interested to determine whether any members of this group represented homologues of additional known *src*-family genes, especially those functioning as growth factor receptors. As we report here, four of these clones, representing overlapping portions of the same genomic segment, hybridized preferentially with the *v-erb-B* oncogene and were further characterized. The deduced amino-acid sequence from a portion of this *Drosophila* genomic segment is 77% homologous to the kinase domain of human epidermal growth factor (EGF) receptor, a substantially greater degree of homology than was observed with any other known *src*-family member. By hybridization with a human EGF receptor complementary DNA clone probe¹⁰, we demonstrate that the same genomic segment showing homology with the kinase domain also contains sequences related to the extracellular domain of the EGF receptor gene.

By screening a *Drosophila* genomic library using hybridization with *v-src* sequences, two types of clones were isolated, exhibiting respectively strong and weak homology with *v-src*. Each of the clones in the former group represented one of the three major *Drosophila src*-related sequences characterized previously⁷⁻⁹. To exclude the possibility that the members of the group showing weak homology to *v-src* were repeat isolates of the previously characterized *Drosophila src*-related sequences, DNA from these clones was hybridized in stringent conditions with probes representing each of the three major *Drosophila src*-related genes; none of the clones reacted with any of these sequences. As the *src* gene family in vertebrates contains many divergent members, we wished to test whether the clones showing low homology to *v-src* contained other known vertebrate *src*-family sequences. The first *src*-family oncogene chosen for secondary screening of these clones was *v-erb-B*, which contains a portion of the avian EGF receptor gene⁶. DNA from each of the 11 clones in the low-homology group was tested by Southern blot¹¹ hybridization with the 0.56-kilobase (kb) *Bam*HI segment of *v-erb-B*¹². This portion of the *v-erb-B* sequence encodes the kinase domain, the most highly conserved region of *src*-family genes. A variable-sized *Eco*RI segment and a 1-kb *Taq*I fragment

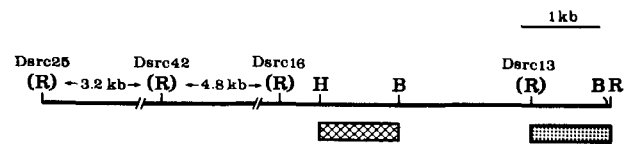


Fig. 1 Restriction endonuclease cleavage map of clones Dsrc13 and Dsrc16. R, *Eco*RI sites; B, *Bam*HI; H, *Hind*III. The *v-erb-B*-complementary *Eco*RI segments of Dsrc13 and Dsrc16 were subcloned into pUC8 for sequencing and restriction mapping. The *Eco*RI sites in parentheses are not genomic sites but are a result of the introduction of linkers during cloning. The dotted box below the map indicates the region that hybridized with *v-erb-B Bam*HI and *Bam*HI/*Eco*RI probes, while the cross-hatched box indicates the region that hybridized with the *Eco*RI/*Nae*I probe specific for the extracellular domain of the human EGF receptor. Hybridizations with the heterologous probes were conducted as follows. Southern blots were pre-hybridized for 3 h at 60 °C in 4×SET (1×SET is 0.15 M NaCl, 0.03 M Tris-HCl pH 8.0, 2 mM EDTA), 10×Denhardt's solution¹⁸, 0.1% SDS and 0.1% sodium PP_i and for an additional hour in the same solution containing denatured calf thymus DNA (Sigma) at 50 µg ml⁻¹. After overnight hybridization in the latter solution in the presence of denatured ³²P-labelled probe, the filters were washed according to the following schedule: 1 h at 60 °C in the hybridization solution; three 45-min intervals at 60 °C in 3×SET, 0.1% SDS and 0.1% sodium PP_i; 45 min at 60 °C in 0.3×SET, 0.1% sodium PP_i, 0.1% SDS and a final wash in 4×SET at room temperature.

from each of the clones Dsrc13, Dsrc16, Dsrc25 and Dsrc42 hybridized more strongly with the *v-erb-B* probe than did DNA from the other seven clones. The 1-kb *Taq*I fragment also hybridized weakly but reproducibly with the 0.54-kb *Bam*HI/*Eco*RI segment of *v-erb-B*, adjacent to and downstream from the 0.56-kb *Bam*HI fragment of *v-erb-B*⁴ (Fig. 1).

Because these clones were isolated by low-stringency hybridization, it was important to determine the nucleotide sequence of a portion of the *Drosophila* DNA so as to demonstrate significant structural homology with the vertebrate EGF receptor gene. The 1-kb *Eco*RI segment of Dsrc13 which hybridized with *v-erb-B* probes was therefore subcloned into plasmid pUC8 and further subcloned into phage M13 mp8. Figure 2 shows the nucleotide sequence and the deduced amino-acid sequence of a portion of the Dsrc13 clone. The *Drosophila* sequence begins at the left end of Dsrc13 (Fig. 1) and corresponds to the cDNA sequence of human EGF receptor¹⁰ at positions 2,383-2,742. This region represents the first 60% of the *v-erb-B Bam*HI fragment used as hybridization probe to identify the *Drosophila* clones.

There are several points of note about the sequence data. First, overall there is 64% nucleotide sequence homology between the two sequences. The longest region of extensive homology, 70%, is from position 166 to the end of the sequence. As the sequence of the *Drosophila* clone further downstream exhibits less homology with the EGF receptor sequence (data not shown), it is apparent that the region shown was responsible for the observed hybridization with the *v-erb-B Bam*HI probe. Second, this region of the avian *c-erb-B* gene is interrupted by at least two introns totalling ~4,600 base pairs¹³. As the *Drosophila* DNA sequence and human cDNA sequences are co-linear, this region of the *Drosophila* gene lacks the two introns present in the avian gene. The structure of the analogous region of the human EGF receptor gene has not been documented. Third, at the amino-acid level, there is 62% homology between the *Drosophila* and human sequences. However, at position 166-360, the amino-acid sequence homology is 77%. It is perhaps noteworthy that the lysine residue at position 295 in the *v-src* protein, which is highly conserved in *src*-family proteins, is also conserved in the *Drosophila* sequence (encoded by nucleotides 37-39, Fig. 2). The analogous amino acid in cyclic AMP-dependent protein kinase is thought to be located in the nucleotide-binding site of the enzyme¹⁴⁻¹⁶.

Because the kinase domain of *src*-family genes is highly conserved, it was important to demonstrate that the homology between the EGF receptor sequence and the Dsrc13 sequence

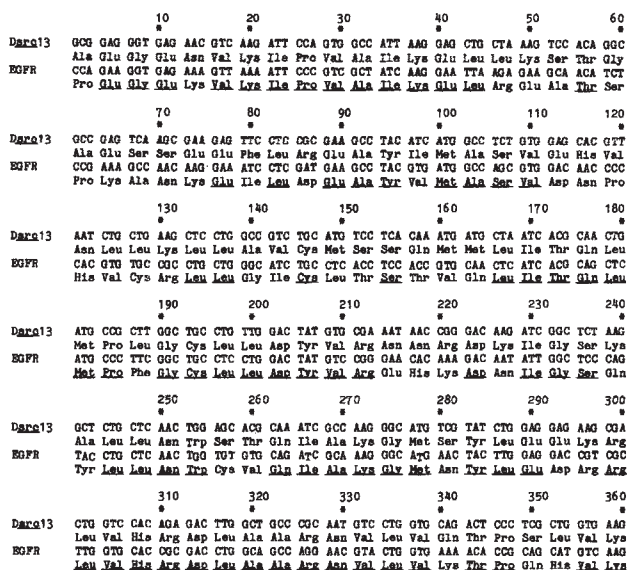


Fig. 2 Nucleotide sequence of a portion of the subcloned *EcoRI* segment from Dsrc13 and the deduced amino-acid sequence. Sequencing was performed by the chain-terminator method¹⁹ using M13 mp8 templates generated by cleavage with *EcoRI*, *MspI* or *Sau3A*. The sequences were aligned and translated using computer programs²⁰. The sequence of the human EGF receptor was taken from ref. 10. Position 1 in the *Drosophila* sequence corresponds to nucleotide 2,383 in the human sequence. Underlined amino acids in the human sequence indicate amino acids conserved in the *Drosophila* sequence.

was significantly higher than that between other *src*-family genes. For this, we selected the region of the *Drosophila* sequence exhibiting the greatest homology with the EGF receptor sequence, nucleotides 166–360. Figure 3 compares the *Drosophila* amino-acid sequence with the sequences of other *src*-family genes. The finding that the homology between the *Drosophila* sequence and the EGF receptor exceeds the next highest comparison by 25% indicates that the Dsrc13 sequence is significantly more closely related to the kinase domain of the EGF receptor than to other known *src*-family genes. Comparisons of the Dsrc13 sequence with *v-raf*, *v-mos* and *v-fgr* are not shown, as the homologies were even lower than those shown in Fig. 3.

The relationship of the cloned *Drosophila* sequences to genomic sequences was defined by a series of Southern blot hybridizations. As noted above, *EcoRI* segments of varying sizes were detected in these clones by *v-erb-B* hybridization, the largest being >12 kb in clone Dsrc25. This suggested that the *EcoRI* site terminating the left-hand end of clone Dsrc13 (as well as Dsrc16 and Dsrc42) was not a genomic site and therefore Dsrc13 and *v-erb-B* probes should hybridize to a genomic *EcoRI* fragment of 12 kb or larger. In addition, as the *v-erb-B* probe detected a *TaqI* fragment common to each of the clones, a similar *TaqI* fragment should be present in genomic DNA. When *Drosophila* genomic DNA restricted with either *EcoRI* or *TaqI* was hybridized in low-stringency conditions with the *v-erb-B* *BamHI* probe

(Fig. 4a), two *EcoRI* segments of 22 and 13.5 kb and two *TaqI* segments of 1 and 0.5 kb were detected. The two *EcoRI* segments hybridized with similar efficiency, while the smaller *TaqI* segment hybridized weakly and is not visible as reproduced here. The 1-kb *TaqI* fragment was equivalent in size to the *TaqI* fragment from each of the Dsrc13, 16, 25 and 42 clones which was also detected by *v-erb-B* hybridization. The same genomic blot was subsequently hybridized with a probe from the subcloned *EcoRI* segment of Dsrc13 (Fig. 1) in high-stringency (Fig. 4b) and low-stringency conditions (Fig. 4c). In high-stringency hybridization conditions, the Dsrc13 probe detected only the 22-kb *EcoRI* segment and the 1-kb *TaqI* segment. Thus, the larger of the two genomic *EcoRI* segments contains the sequences represented by the overlapping set of clones that we have identified by *v-erb-B* hybridization. In low-stringency hybridization conditions, the Dsrc13 probe still hybridized primarily with the larger of the *EcoRI* and *TaqI* bands, although weak hybridization to the *v-erb-B*-complementary 13.5-kb *EcoRI* segment and at least one additional *EcoRI* segment was observed. These results suggest that there is a single-copy genomic segment represented by the set of overlapping clones Dsrc13, 16, 42 and 25 identified by hybridization with *v-src* and *v-erb-B* probes. The presence of additional genomic sequences showing homology to *v-erb-B* is suggested by the smaller *EcoRI* segments that hybridized with the *v-erb-B* and Dsrc13 probes in less stringent conditions. We believe that some of the additional genomic sequences detected by *v-erb-B* hybridization represent *Drosophila src*-family sequences related to the kinase domain of the *v-erb-B* oncogene. Confirmation of this hypothesis awaits the isolation of the remainder of the *v-erb-B*-complementary genomic segments.

Although the above results documented a degree of homology between Dsrc13 and the cytoplasmic domain of the human EGF receptor, we could not conclude that the *Drosophila* sequences represented a true homologue of the vertebrate gene. In addition to the kinase domain, the EGF receptor contains a short transmembrane domain and a large extracellular domain that is responsible for EGF binding¹⁰, and it was important to determine whether other portions of the vertebrate gene were represented on one or more of our *Drosophila* clones. An *EcoRI*/*NaeI* fragment from the human EGF receptor cDNA clone was isolated as a hybridization probe specific for the extracellular domain of EGF receptor; this fragment starts at position 293 from the extreme 5' end of the amino-acid-coding region and stops short of the transmembrane domain by 100 nucleotides¹⁰. Hybridization of clones Dsrc16, 42 and 25 with the *EcoRI*/*NaeI* probe revealed the same *EcoRI* segments that hybridized with the *v-erb-B* *BamHI* probe (data not shown, see Fig. 1). These *EcoRI* segments vary in size due to the introduction of *EcoRI* linker sites during construction of the library¹⁷. DNA from clone Dsrc13 did not hybridize with the *EcoRI*/*NaeI* probe, a not unexpected finding considering the sequence and arrangement of this clone. To characterize further the homology of the *EcoRI*/*NaeI* probe to *Drosophila* sequences, the pattern of hybridization to cloned and genomic sequences was determined. In the cloned DNA, *EcoRI*/*NaeI* probe hybridization was limited to a 1-kb *HindIII*/*BamHI* segment (Fig. 4d). The pattern of *EcoRI*/*NaeI* probe hybridization to genomic DNA

Fig. 3 Alignments of deduced amino-acid sequences of Dsrc13, EGF receptor and other *src*-family members. The sequence of Dsrc13 for amino acids 56–120 was aligned with the analogous region of various *src*-related proteins. The gaps in some of the sequences were introduced manually to achieve maximal alignment of the most highly conserved regions. Only the matches between Dsrc13 and EGF receptor are boxed. Positions in the other sequences that match the Dsrc13 sequence are shown in capital letters. Lower-case letters in the Dsrc13 sequence indicate that no other sequence matches at that position. The numbers on the right-hand side indicate the homology of that sequence with Dsrc13. The sources of the sequence data were as follows: Dash, ref. 7; *v-abl*, ref. 7; Dsrc4, ref. 9; *v-fes*, ref. 21; *v-fps*, ref. 22; *v-src*, ref. 23; Dsrc, ref. 7; *v-yes*, ref. 24; *v-fms*, ref. 25.

LIETQLMPLICLLDLYVnrrnRkIG	LIETQLMPLICLLDLYVnrrnRkIG	S KALLNWSIKIAKQMSYLRRLVRRDLAARNVLYVQFRLV	S KALLNWSIKIAKQMSYLRRLVRRDLAARNVLYVQFRLV	Dsrc13	775
ITETMhKGLVDFLIRaagret1	davALLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	Dsrc13	525
ITETMhKGLVDFLIRaagret1	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	Dsrc13	515
ITETMhKGLVDFLIRaagret1	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	Dsrc13	465
ITETMhKGLVDFLIRaagret1	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	Dsrc13	405
ITETMhKGLVDFLIRaagret1	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	Dsrc13	375
ITETMhKGLVDFLIRaagret1	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	Dsrc13	375
ITETMhKGLVDFLIRaagret1	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	Dsrc13	375
ITETMhKGLVDFLIRaagret1	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	Dsrc13	375
ITETMhKGLVDFLIRaagret1	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	Dsrc13	375
ITETMhKGLVDFLIRaagret1	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	SAVLLYmaTQIAaGMSLSErnyIHRDLAARNVLYVgdnLVK	Dsrc13	375

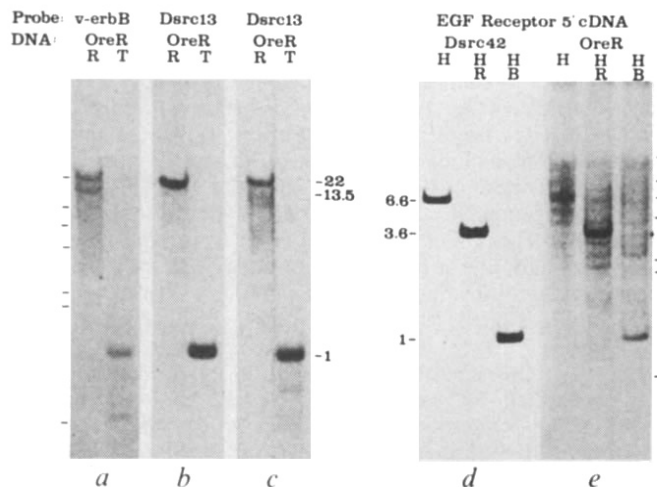


Fig. 4 Southern blot hybridization analysis of cloned and genomic sequences. In *a*–*c*, Southern blots of Oregon R genomic DNA cleaved with either *EcoRI* (R) or *TaqI* (T) were hybridized with the *v-erbB* *Bam*HI fragment probe or the *Dsrc13* *EcoRI* fragment probe. In *d* and *e*, respectively, Southern blots of whole phage DNA from the *Dsrc42* clone (see Fig. 1) or Oregon R genomic DNA cleaved with *HindIII* (H), *HindIII* plus *EcoRI* (HR) or *HindIII* plus *Bam*HI (HB) were hybridized with the *EcoRI*/*NaeI* probe specific for the extracellular domain of the human EGF receptor cDNA clone. With the exception of *b*, hybridizations were conducted as described in Fig. 1 legend; for *b*, the hybridization conditions were identical except that the temperature of hybridization was 67 °C. The dashes on the left and right sides of the figure indicate the positions of λ DNA cleaved with *HindIII*. The approximate sizes of the hybridizing DNA fragments noted in the text are also shown.

was essentially the same as in the cloned DNA (Fig. 4*e*). In each digest, the unique band of hybridization in the cloned DNA corresponded in size to the major band of hybridization in genomic DNA. We therefore conclude that the major genomic sequence detected by hybridization with the extracellular domain probe of EGF receptor is located on the same cloned DNA segment that has homology with the kinase domain of the EGF receptor. Additional nucleotide sequencing experiments are in progress to confirm the identity of the sequences in the region of our *Drosophila* clones which hybridize with the *EcoRI*/*NaeI* probe. Hybridization of the *EcoRI*/*NaeI* probe to additional genomic sequences was also observed (Fig. 4*e*), but their nature is unknown. However, the *v-erbB* probe also detected genomic sequences not present on our cloned DNA segments (Fig. 4*a*). Taken together, these results suggest that other genomic sequences related to both domains of the EGF receptor may be responsible for the observed hybridization.

As the *Dsrc13* probe contains sequences from an amino-acid-coding region and does not contain repeated sequences detectable in high-stringency hybridization conditions (Fig. 4*b*), this probe was used to test for RNA expression of the putative EGF receptor gene, and readily revealed a poly(A)-containing transcript of 6 kb in embryos, pupae and adults (Fig. 5). In pupal RNA, this band seems to be a closely spaced doublet. These results suggest that the sequences exemplified by the *Dsrc13* clone represent a functional *Drosophila* gene.

In vertebrate cells, the mitogenic activity of EGF is transmitted through the EGF receptor. Because of its different developmental programme, the study of these sequences in *Drosophila* offers a different perspective of EGF receptor function. For the first 2 h of *Drosophila* development, there are virtually continuous rounds of chromosome and nuclear divisions although there are no cells and no cell divisions. At the blastoderm stage, the fate of cells is determined such that some cells, the imaginal cells, will divide mitotically until the adult is formed during metamorphosis. Other cells that give rise to larval tissues will

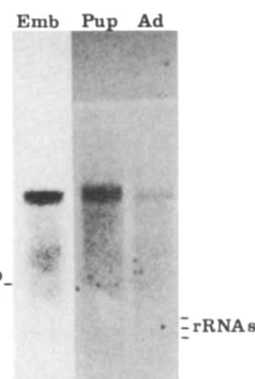


Fig. 5 RNA expression of *Dsrc13* sequences. Approximately 5- μ g samples of poly(A)-containing RNA from an 18-h collection of *Drosophila* embryos (Emb), mid-metamorphic stage animals (Pup) and adult flies (Ad) were electrophoresed through formaldehyde/agarose gels, blotted and hybridized with *Dsrc13* probe as described previously²⁶. The positions of the rRNA species (2 kb, 1.9 kb and 1.7 kb) were determined by staining of the nitrocellulose filter. The position of the *hsp83* mRNA (3.05 kb) was determined by hybridization with cloned *hsp83* gene sequences²⁷.

not divide during the 4 days of larval life although chromosome duplication does continue. Thus, there are fluctuations in the mitotic states of cells in *Drosophila* that have no counterpart in vertebrate systems. We plan to exploit these differences to enhance our understanding of EGF receptor structure and function and its involvement in *Drosophila* development.

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Investigation of genetic linkage between myosin and actin genes using an interspecific mouse back-cross

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The introduction of cloned probes to follow the segregation of DNA restriction fragment length polymorphisms (RFLPs) has led to a revival of mendelian genetics in attempts to map the human genome¹. In the mouse, however, it has often proved difficult to detect an RFLP with a DNA probe between different inbred strains of the laboratory mouse. To circumvent this problem, we have used two species, *Mus musculus domesticus* and *Mus spretus*² which interact as sympatric species³ but can be interbred under laboratory conditions⁴. Because of the relative evolutionary distance between these species, they exhibit polymorphism at many more loci than do different strains of the usual *M. m. domesticus* laboratory mouse². This is also observed at the DNA level when the sizes of restriction fragments encoding a specific gene are compared⁵. We have used these RFLPs between *M. m. domesticus* and *M. spretus* to follow the segregation of genes encoding different isoforms of myosin alkali light chains in the backcross progeny between these species and to compare this with that of other contractile protein genes. No linkage between these genes was observed.

We have characterized several DNA sequences which serve as specific probes for genes encoding different isoforms of myosin alkali light chains in the mouse, including the isoforms of fast skeletal muscle (MLC1_F/MLC3_F)⁵, cardiac ventricular muscle (MLC1_V), which is also the major isoform expressed in slow skeletal muscle (MLC1_S)⁶, and cardiac atrial muscle (MLC1_A), also expressed in fetal skeletal and ventricular muscle (MLC1_{emb})^{7,8}. The two light chains, MLC1_F and MLC3_F, are encoded by a single functional locus, from which a processed pseudogene has been generated in some mouse species (for example, *Mus musculus*); this is absent from *M. spretus*⁵. The other isoforms are encoded by unique loci^{6,8}. These probes reveal fragments of different sizes in the DNA of inbred lines of *M. spretus* (SPE/Pas) and of *M. musculus* (DBA/2) on restriction with many different enzymes, which thus characterize allelic forms of the genes.

The segregation of the myosin light-chain (MLC) alleles in 42 offspring from a back-cross is presented in Table 1 and analysed in Table 2. An example of such an experiment with the MLC1_A (MLC1_{emb}) gene is shown in Fig. 1. There is no co-segregation of the genes encoding MLC1_F/MLC3_F, MLC1_A (MLC1_{emb}) or MLC1_V (MLC1_S), thus, there is no genetic linkage. In this backcross analysis, linkage would have been detected for genes up to 30 centimorgans (cM) apart, with a 99% confidence limit⁹. Twenty-one previously localized polymorphic markers¹⁰, covering about half of the mouse genome, have been analysed for their segregation in the same back-cross. Comparison with the segregation of the myosin light-chain genes demonstrates linkage of the gene for MLC1_F/MLC3_F to *Idh-1* (chromosome 1), for MLC1_V (MLC1_S) to *Es-14* (chromosome 9) and for MLC1_A (MLC1_{emb}) to *Es-3* (chromosome 11). Therefore, these myosin light-chain genes are located on different mouse chromosomes.

The localization of the MLC1_F/MLC3_F gene was confirmed by the analysis of B × D recombinant inbred strains derived from

Table 1 Segregation of the contractile protein genes and of biochemical markers in the back-cross

	Animal no.																					Chromosomal assignments of markers
Locus	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
MLC1 _F /MLC3 _F	-	+	+	+	+	-	-	-	-	+	+	-	-	+	+	+	+	-	-	-	-	1
<i>Idh-1</i>	-	+	+	+	+	-	-	-	-	+	+	-	-	+	+	+	+	-	-	ND	-	
MLC1 _V	-	-	+	-	+	+	+	+	-	+	+	-	-	-	-	-	+	+	-	+	+	
<i>Es-14</i>	-	-	+	-	+	+	+	+	-	+	+	-	-	-	-	-	+	+	-	ND	+	9
MLC1 _A	-	+	-	-	-	+	-	-	+	-	+	+	+	+	-	+	-	+	+	-	+	
<i>Es-3</i>	+	+	-	-	-	+	-	-	+	-	+	+	+	+	-	+	-	+	+	ND	+	
MHC _F	-	+	+	-	+	-	+	-	+	+	+	+	+	-	-	+	-	+	+	-	+	11
<i>Hba</i>	+	-	+	ND	+	-	+	-	+	+	+	-	+	-	ND	ND	-	+	-	-	ND	
α -Cardiac actin	+	-	-	-	+	-	+	-	+	-	+	+	-	+	+	+	-	-	+	-	+	
α -Skeletal actin	+	+	-	+	-	-	+	-	+	-	+	+	-	-	-	+	+	+	-	+	+	
	Animal no.																					Chromosomal assignments of markers
Locus	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	
MLC1 _F /MLC3 _F	+	-	+	+	-	-	+	-	+	+	-	+	-	+	+	-	-	+	+	-	-	1
<i>Idh-1</i>	+	-	+	-	-	-	+	+	+	+	-	+	-	+	+	-	-	ND	ND	ND	ND	
MLC1 _V	-	-	-	-	+	-	-	-	+	-	+	+	-	+	-	-	+	+	-	+	+	
<i>Es-14</i>	-	-	-	-	+	-	-	-	+	-	+	+	-	+	-	-	+	ND	ND	ND	ND	9
MLC1 _A	-	+	+	+	+	-	+	-	-	+	-	-	-	+	+	+	-	-	-	+	-	
<i>Es-3</i>	-	+	+	-	+	-	+	+	-	+	-	-	-	+	+	+	-	ND	ND	ND	ND	
MHC _F	+	+	+	+	-	-	+	+	-	+	-	-	-	+	+	+	-	+	-	+	+	11
<i>Hba</i>	+	ND	ND	+	-	+	-	ND	ND	ND	-	-	-	+	-	-	+	+	+	ND	ND	
α -Cardiac actin	+	-	+	+	+	-	-	-	-	+	+	+	-	+	+	+	-	-	+	-	+	
α -Skeletal actin	-	-	+	-	+	+	-	-	+	-	-	+	-	+	-	+	-	-	-	+	-	11

The animals are scored as homozygous for the *musculus* alleles (-) or heterozygous (+). ND, not determined. Animals 1-20 are females, 21-42 are males. *Idh-1*, *Es-3* and *Es-14* were analysed as described in ref. 20. The segregation of *Hba* was analysed using a *Hind*III RFLP detected with an α -globin cDNA²¹; see Fig. 1. Similarly, the MLC1_F/MLC3_F gene was analysed using *Eco*RI or *Bcl*I (blot washing at 45 °C), the MLC1_V (MLC1_S) gene using *Bam*HI (blot washing at 45 °C), the MHC_F gene using *Bgl*II (blot washing at 72 °C), the α -cardiac actin gene using *Bcl*I or *Sac*I (blot washing at 65 °C) and the α -skeletal actin gene using *Xba*I or *Bgl*II (blot washing at 72 °C). The MLC1_A (MLC1_{emb}) gene was analysed using *Bam*HI as illustrated in Fig. 1.

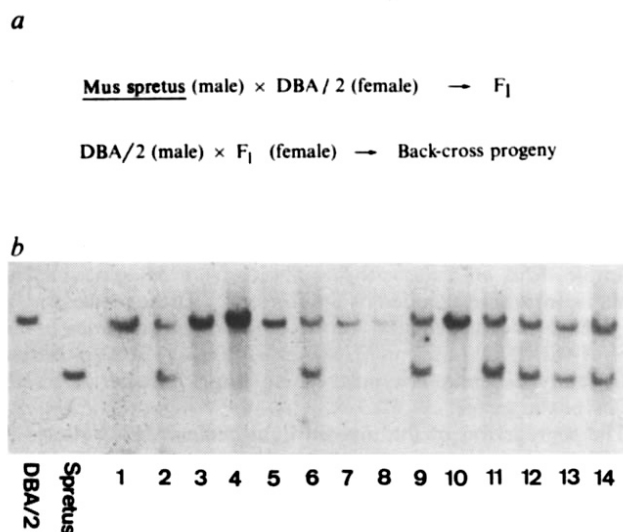


Fig. 1 *a*, Outline of the backcross strategy between inbred strains of *M. m. domesticus* (DBA/2) and *M. spretus* (SPE/Pas). 42 offspring were analysed for the segregation of RFLPs and 37 for the segregation of biochemical markers. The F_1 males are sterile²² because of a limited number of loci (J.-L.G., unpublished results). *b*, An example of the segregation of the RFLPs for the $MLC1_A$ ($MLC1_{emb}$) gene. DNA was extracted from the spleen of the parental animals and of the offspring, restricted and analysed on blots as described in ref. 23; 5 μ g of each DNA sample were restricted with *Bam*HI and blotted onto nitrocellulose. Blots were probed with the $MLC1_A$ ($MLC1_{emb}$) cDNA labelled by nick-translation as described in ref. 23, then washed in $0.1 \times$ SSC at 65 °C before autoradiography. Lanes DBA/2 and Spretus correspond to the parental DNA samples, lanes 1 to 14 to the first 14 back-crosses analysed.

C57BL/6J (B) and DBA/2J (D) *M. m. domesticus* parental strains⁹, in which allelic forms of *Idh-1* segregate¹⁰ (Fig. 2). Of 24 recombinant inbred strains analysed, only two do not show a co-segregation between $MLC1_F/MLC3_F$ and *Idh-1*, indicating a genetic distance of ~ 2 cM between these loci⁹. Similarly, in B $\times$ H recombinant inbred strains derived from C57BL/6J (B) and C3H/HeJ (H)⁹, the $MLC1_A$ ($MLC1_{emb}$) locus co-segregates with *Glk* and, with one exception, with *Es-3* (Fig. 2).

The segregation of the genes encoding the α -skeletal and α -cardiac actins^{11,12} and a fast skeletal muscle isoform of the myosin heavy chain (MHC_F)¹³ was analysed in the same back-cross. Our results confirm that these genes segregate independently, as expected from the analysis of somatic cell hybrids, which allocates them to different mouse chromosomes¹⁴⁻¹⁶. In addition, we show that they are not linked to myosin light-chain genes in the mouse genome, except for a loose linkage (70% co-segregation) between the MHC_F and $MLC1_A$ ($MLC1_{emb}$) loci, which are both allocated to chromosome 11 (Tables 1, 2). This linkage suggests that the MHC_F gene lies in the middle of

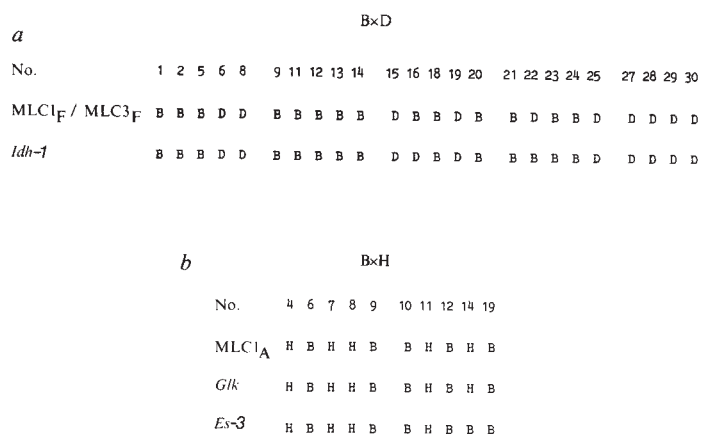


Fig. 2 *a*, Co-segregation of the $MLC1_F/MLC3_F$ and *Idh-1* loci in the B $\times$ D recombinant inbred strains. *b*, Co-segregation of the $MLC1_A$ ($MLC1_{emb}$), *Glk* and *Es-3* loci.

Methods. *a*, A *Pst*I RFLP was defined for the myosin light-chain locus between the parental strains. DNA samples of the 24 recombinant inbred strains were purchased from the Jackson laboratory (Bar Harbor) and analysed as described in Fig. 1 legend. *b*, A *Hinf*I RFLP for the *MLC* locus was used in the same way to follow the segregation of the gene in the 10 B $\times$ H recombinant inbred strains. The segregation pattern of *Idh-1*, *Glk* and *Es-3* in these recombinant inbred strains is described in ref. 10.

chromosome 11 and that the genes are arranged in the order *Hba/MHC_F/MLC1_A/Es-3* from the centromere on this chromosome.

We conclude, therefore, that the co-expression of contractile protein genes in an adult or fetal, skeletal or cardiac muscle¹⁷ does not require a structural proximity of these genes. Furthermore, the genes coding for different alkali myosin light-chain isoforms, which have probably arisen by gene duplication, have been dispersed in the mouse genome, in contrast to some other multigene families such as that of the β -globin genes¹⁸.

This represents the first report of gene mapping based on the analysis of RFLP segregation in crosses between different mouse species. It is important to show that the evolutionary distance between them does not introduce a bias. For the following reasons, we argue that this is not the case. First, no gross reorganization of the chromosomes has been observed between these species by cytological methods (H. Winking, personal communication). The fact that they breed and give rise to viable and fertile offspring supports this observation. Second, the genes linked on the mouse genetic map co-segregate in our crosses and those which are not linked do not (for example, there are only 8/37 recombinants between *Amy-1* and *Adh-1* (chromosome 3) and 8/37 between *Pgm-1* and *Alb-1* (chromosome 5); see also ref. 19). In addition, our localizations for the $MLC1_F/MLC3_F$ and $MLC1_A$ ($MLC1_{emb}$) genes have been confirmed using recombinant inbred strains.

The type of analysis presented here has several advantages over the use of recombinant inbred strains: (1) The use of

Table 2 Analysis of the segregation of alleles in the back-cross

	$MLC1_A$	$MLC1_V$	α -Skeletal actin	α -Cardiac actin	MHC_F	<i>Idh-1</i>	<i>Es-3</i>	<i>Es-14</i>	<i>Hba</i>
$MLC1_F/3_F$	20/42	19/42	22/42	24/42	23/42	35/37	15/37	18/37	18/31
$MLC1_A$		15/42	26/42	26/42	31/42	16/37	34/37	13/37	12/31
$MLC1_V$			21/42	17/42	18/42	18/37	12/37	37/37	17/31
α -Skeletal actin				26/42	21/42	18/37	22/37	18/37	14/31
α -Cardiac actin					25/42	19/37	23/37	16/37	16/31
MHC_F						21/37	27/37	14/37	21/31
<i>Idh-1</i>							17/37	18/37	14/28
<i>Es-3</i>								12/37	11/28
<i>Es-14</i>									16/28

Numbers refer to the numbers of co-segregants per total number of animals analysed.

back-crosses extends the analysis over longer distances (30 cM), whereas recombinant inbred strains tend to keep linked only those loci that are very close together⁹. (2) During the generation of recombinant inbred strains, certain associations of genes may be favoured or selected against, resulting in a nonrandom segregation of loci not observed in a back-cross. (3) The use of distantly related species increases the probability of finding an RFLP for a given gene, so that any gene for which a complementary DNA (cDNA) is available can be analysed.

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A test for intron function in the yeast actin gene

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Many eukaryotic genes contain intervening sequences (IVS)^{1,2}, but the rationale for their existence remains a mystery. Previous studies done in our laboratory demonstrated that the intron in a yeast tRNA^{Tyr} gene, *SUP6*, does have a function^{3,4}. We used the same approach to determine the role of introns in nuclear genes encoding messenger RNAs. A single actin gene with one intron exists in *Saccharomyces cerevisiae*^{5,6}. The level of actin in yeast appears to be crucial to viability: either too much or too little actin inhibits growth⁷. Therefore, small effects on synthesis of actin protein resulting from the removal of the actin gene intron would be expected to cause measurable changes in cell growth. In the present study, an intron-deleted actin gene was constructed *in vitro* and was used to replace the single resident actin gene in a haploid strain. Analysis of the cells carrying the intron-deleted actin gene shows that the intervening sequence is not essential for actin gene expression.

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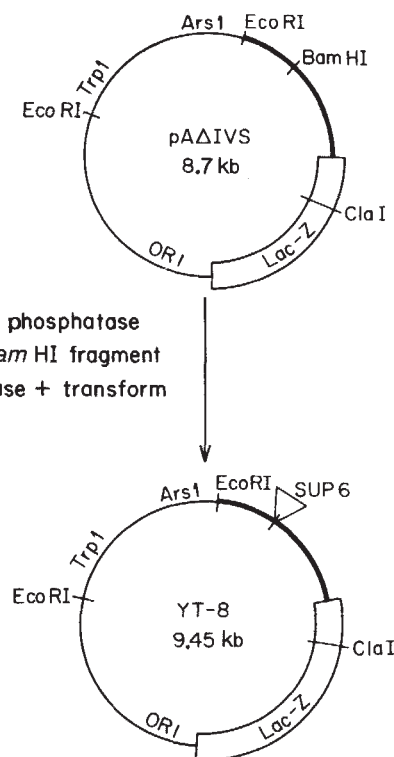


Fig. 1 Construction of plasmid YT-8. Construction of the actin/ β -galactosidase fusion plasmid with the actin intron removed (pAΔIVS) has been reported elsewhere⁸. Plasmid YT-8 was constructed by inserting a 0.75-kb *Bam*HI fragment containing the *SUP6* tRNA gene into the *Bam*HI site of pAΔIVS. The thick line indicates the segment containing the yeast actin gene and its flanking sequences.

Deletion of the intron from a yeast actin/ β -galactosidase fusion gene (in pAΔIVS) has been reported previously⁸; this plasmid was modified further by inserting a 750-base pair (bp) *Bam*HI fragment containing the *SUP6* tRNA gene into the unique *Bam*HI site upstream of the actin gene (Fig. 1). *SUP6*, a tyrosine-inserting ochre suppressor, provides a selectable marker for chromosomal integration (Fig. 1) through selection for Leu⁺ and His⁺ transformants by *SUP6* suppression of the ochre mutation in those genes. Plasmid DNA from YT-8 (plasmid pAΔIVS with the *SUP6* gene inserted in the *Bam*HI site) was digested with the restriction enzymes *Eco*RI and *Cla*I, then used to transform the haploid yeast strain DBY874 [*α his5-2* (oc), *leu2-1* (oc), *can1-100* (oc), *ura3-52*, *trp5-48* (oc)]. Transformants were initially selected on the basis of their ability to grow on leucine-deficient media. To distinguish from possible Leu⁺ revertants, the candidates were rescreened for their ability to grow on histidine-deficient media. An alteration of the actin locus could be generated by either genetic recombination (a double crossover between the incoming fragment and the homologous region on the actin gene) or gene conversion (heteroduplex formation between the incoming actin sequences and genomic DNA at the actin locus); either of these mechanisms, or a combination of the two, can be invoked to explain the changes observed in the recombinant actin genes⁹.

To determine whether an intron-deleted actin gene had been created by this procedure, genomic DNAs from candidate transformants and from the untransformed parent were compared by Southern blot analysis (Fig. 2). When wild-type yeast DNA is digested with *Eco*RI and *Hind*III, two fragments of 1.6 and 2.2 kilobases (kb) are generated which hybridize with a radioactive actin-specific probe^{5,6}. As the 1.6-kb fragment contains only the 3' half of the actin gene, it will remain unchanged in the transformants. The 2.2-kb fragment includes the 5'-flanking

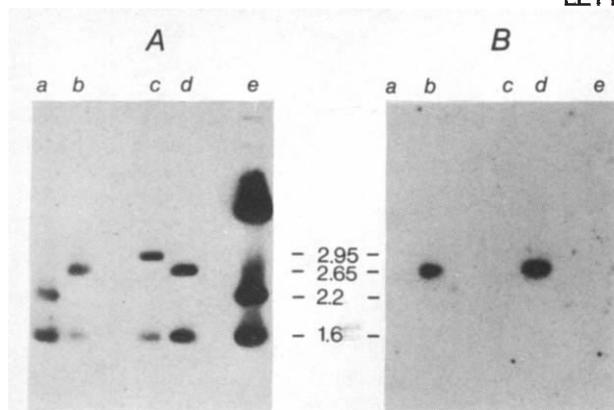


Fig. 2 Southern blot analysis of genomic DNA from DBY874 and transformants 513-A, -V and -L. **A**, Genomic DNA was digested with the restriction enzymes *EcoRI* and *HindIII*. After electrophoresis in a 1% agarose gel, DNA was transferred to a nitrocellulose filter and hybridized with nick-translated pYactI (3.8-kb *EcoRI* fragment containing the actin gene in pBR322; refs 5, 6). All tested strains have the smaller 1.6-kb *EcoRI/HindIII* fragment in common, whereas the size of the larger *EcoRI/HindIII* fragment (upper band) differs: **a**, DBY874, 2.2 kb (same size as the wild-type fragment in pYact (**e**)); **b**, **d**, 513-A and 513-V, respectively, 2.65 kb (insertion of the *SUP6* gene and deletion of the actin intron); **c**, 513-L, 2.95 kb (insertion of the *SUP6* gene, but with the actin intron present). **B**, The filter in **2A** was rehybridized with a [32 P]-labelled 17-base oligonucleotide specific to the actin exon-exon junction.

region and a large portion of the actin gene; the size of this band will vary in transformants because it contains the inserted *SUP6* gene and the actin intron region. Two different classes of transformants were found based on the size of the larger *HindIII/EcoRI* fragment (Fig. 2A): (1) a 2.95-kb band (in 513-L, which has the *SUP6* gene inserted upstream of the actin gene but still contains the actin intron) and (2) a 2.65-kb band (in 513-A and 513-V, which have the *SUP6* gene inserted and no longer contain the intron). No plasmid sequences were present in the transformants (data not shown). Definitive proof of the removal of the actin intron was obtained by hybridization of genomic DNA digested with *HindIII* and *EcoRI* with a [32 P]-labelled 17-base oligonucleotide specific to the actin exon-exon junction⁸. Only the intron-deleted strains hybridized with the labelled oligonucleotide (Fig. 2B).

Expression of the intron-deleted actin gene was analysed by Northern blot analysis of actin-specific mRNA from the wild-type strain and from transformed strains (Fig. 3). Using a nick-translated restriction fragment specific for the actin intron, no actin precursor mRNA could be detected in RNA prepared from the transformant 513-A, whereas it was present in RNA from wild-type strains and from the strain 513-L (Fig. 3A). Hybridization of actin-specific DNA, labelled by nick-translation, with poly(A)⁺ RNA showed that the relative amounts of actin mRNA were roughly equivalent in the strains with and without the actin intron and *SUP6* gene (Fig. 3B); however, the level of actin has not been analysed. Further, the strains 513-A and 513-V grew at a rate similar to that of their counterpart 513-L (their doubling times are within 5% of each other).

As the level of actin protein is critical for cell survival, strains 513-A and 513-V must have normal amounts of actin protein⁷. Thus, the deletion of the intervening sequence does not affect the level of actin mRNA in the cell. However, small differences in the level of mRNA and/or actin cannot be ruled out. In addition, there is no apparent effect on sporulation. Diploid cells homozygous for the intron-deleted actin gene sporulate to

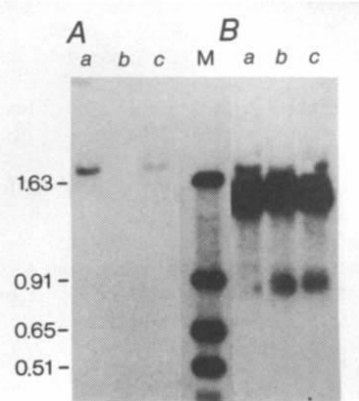


Fig. 3 Northern blot analysis of poly(A)⁺ RNA from DBY874 and transformants 513-A and 513-L. **A**, Hybridization of poly(A)⁺ RNA with a nick-translated actin intron-specific fragment (204-bp *XhoI/ClaI* fragment of pYact I^{5,6}). Actin precursor mRNA (~1,700 nucleotides) is detected only in DBY874 (**a**) and the transformant 513-L (**c**). No actin precursor mRNA is found in strain 513-A (**b**). **B**, The filter used in **A** was rehybridized with nick-translated actin DNA (3.8-kb *EcoRI* fragment of pYact I). Actin mRNA (~1,400 nucleotides) is present in similar amounts in all strains tested. (The probe pYact I also contains a region which hybridized with RNA from the YP2 gene, 800 nucleotides long^{11,12}.)

Methods. RNAs from DBY874 (**a**), 513-A (**b**) and 513-L (**c**) were isolated from cells grown to mid-logarithmic phase ($A_{600} = 2-5$) by hot phenol extraction. Poly(A)⁺ RNA was obtained by oligo(dT)-cellulose chromatography. Glyoxylated poly(A)⁺ RNA (10 μ g) from each strain was electrophoretically separated on a 1.8% agarose gel in 10 mM phosphate buffer¹³. Nucleic acids were transferred to nitrocellulose filters and hybridized according to the procedure of Thomas¹⁴. Lane M corresponds to 5'-labelled *AluI* and *HinfI* fragments of pBR322 serving as size markers.

generate four viable spores (R. Parker, personal communication).

We conclude from this experiment that the intron in the yeast actin gene does not have an observable function. It is possible that the role of the intron is too subtle to be observed in laboratory conditions of growth or that the intron, while having evolutionary significance, has no present role. To conclude that this is true for all yeast genes that contain introns would of course be premature, but there exist strains in which mitochondrial introns have been removed¹⁰ with no observable effect.

Insertion of a selectable marker upstream of the gene of interest may serve as a general method for efficiently transferring mutations into the yeast genome.

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Does *Paramecium primaurelia* use a different genetic code in its macronucleus?

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It has long been known that messenger RNAs (mRNAs) of ciliates and in particular of *Paramecium* are not translated well in heterologous *in vitro* translation systems¹⁻⁴. Recently, we have demonstrated for *Paramecium primaurelia* that this phenomenon results from the presence of well-defined blocking sites in the coding sequences of almost all mRNAs, and that these sites are an intrinsic feature of the primary as opposed to the secondary structure of the mRNAs⁵. Here we show that both the gene and the mRNA for the G surface antigen of *P. primaurelia* contain numerous TAA and TAG codons scattered throughout their coding sequences. We propose that these codons do not represent termination codons in *P. primaurelia* but instead code for glutamic acid or glutamine and that the *in vitro* translation of *Paramecium* mRNAs is blocked by their presence.

The G surface antigen is one of the set of surface antigens expressed by *P. primaurelia* in a mutually exclusive manner^{6,7}. It is a highly abundant protein covering the whole surface of the cell, and comprises a unique polypeptide chain of relative molecular mass 250,000–300,000 (refs 8, 9). Its mRNA is ~8 kilobases (kb) long and can be detected readily on an RNA gel^{4,5,10}. From a genomic library of macronuclear DNA, two

EcoRI fragments of 5.6 and 1.2 kb, EG1 and EG2, respectively, were selected⁵. Direct proof that the EG1 and EG2 fragments contain sequences coding for the G surface antigen was obtained by selectively hybridizing them with G-antigen mRNA^{3,10} and by the direct expression in *Escherichia coli* of subfragments of EG1 and EG2 sequences (E.M., F.C. and A. Baroin, in preparation, and see below for EG2): at least two subfragments, shown in Fig. 1, code for antigenic determinants of the G antigen.

Southern blotting of genomic DNA shows that the G-antigen gene is situated between a *Bam*HI site and the end of a macronuclear chromosome 15.5-kb away; this region is unique in the genome (its restriction map is given in Fig. 1). The gene is oriented with its 3' end towards the end of the chromosome. By Northern blotting of total RNA from G-expressing paramecia, EG2 is shown to be the most downstream *EcoRI* fragment of the macronuclear chromosome hybridizing to the mRNA of the G antigen (not shown). The precise location of the 3' end of the transcript was determined by S₁ mapping (not shown) and it is found to lie 70 bp downstream from the *EcoRV* site of EG2 (see Fig. 1). Upstream from this point, the EG2 sequence is completely protected by the mRNA in S₁ protection experiments (not shown).

The entire EG2 fragment was sequenced by the Maxam-Gilbert technique¹¹. The strategy used for sequencing is shown in Fig. 1, as is the noncoding strand. This sequence is remarkable in that two regions can be distinguished clearly on the basis of their A+T content (see Fig. 1): the A region at the 5' end (the first 1,137 base pairs, bp) contains 62% A+T with a marked 3-bp periodicity in their distribution (see Fig. 2b) whereas the last 105 bp at the 3' end (the B region) yields the significantly higher value of 82% A+T and no such periodicity. In organisms having A+T-rich genomes, coding regions usually have a much lower A+T content than noncoding regions and most

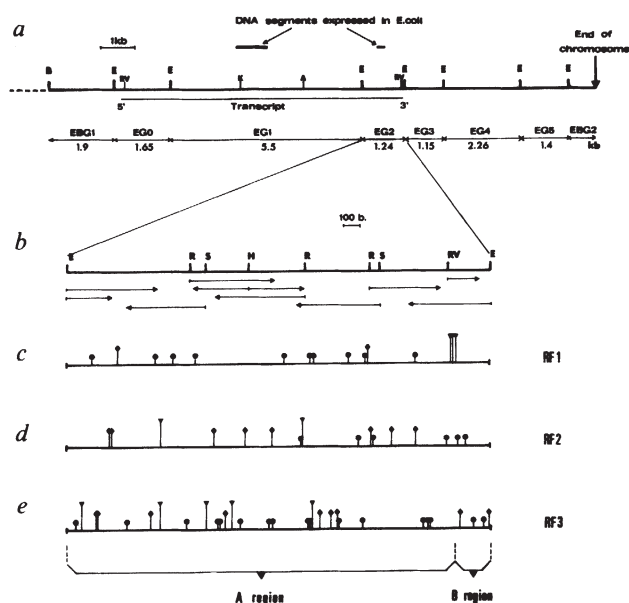


Fig. 1 Structure of the G surface antigen gene and sequence of its 3' end. **a**, Map of the region of the macronuclear chromosome containing the G surface antigen gene (E, *EcoRI*; B, *Bam*HI; RV, *EcoRV*; A, *Ava*I; K, *Kpn*I; H, *Hind*II; S, *Sau*3A). After addition of a synthetic *Bam*HI linker to the end of the macronuclear chromosome, we were able to clone the 15.5-kb *Bam*HI fragment in λ EMBL3 (ref. 24). The different *EcoRI* fragments of the cloned 15.5-kb fragment, named (from left to right) EBG1, EG0, EG1, EG2, EG3, EG4, EG5 and EBG2, were subcloned separately in plasmid pUC12 or pBR328. The position of the transcript was determined by S₁ mapping according to ref. 25: the 5' end is localized in the EG0 fragment, and the 3' end in the EG2 fragment. The EG2 fragment, which contains the 3' end of the message, was sequenced (**f**) by the Maxam and Gilbert procedure¹¹; the sequencing strategy is shown in **b**. **c–e**, The positions of the stop codons in the three possible reading frames: $\bullet$, TAA; $\blacklozenge$, TAG; and $\blacktriangledown$, TGA. On the basis of % A+T content, two regions can be distinguished: the A region with 62% A+T and the B region with 82% A+T. **f**, The EG2 sequence. The 1,242-base DNA strand shown is the noncoding strand and the reading frame is RF1. The dotted line indicates the part of the EG2 sequence that is not protected by the mRNA; the dashed line indicates the DNA subfragment containing an antigenic determinant of the G surface antigen (see Fig. 3); solid line, the RNA sequence determined from the *Sph*I/*Sph*I fragment by the primer extension method (see Fig. 4). A cluster of three TGAs near the 3' end (boxed) is located at the junction between the A and B regions.

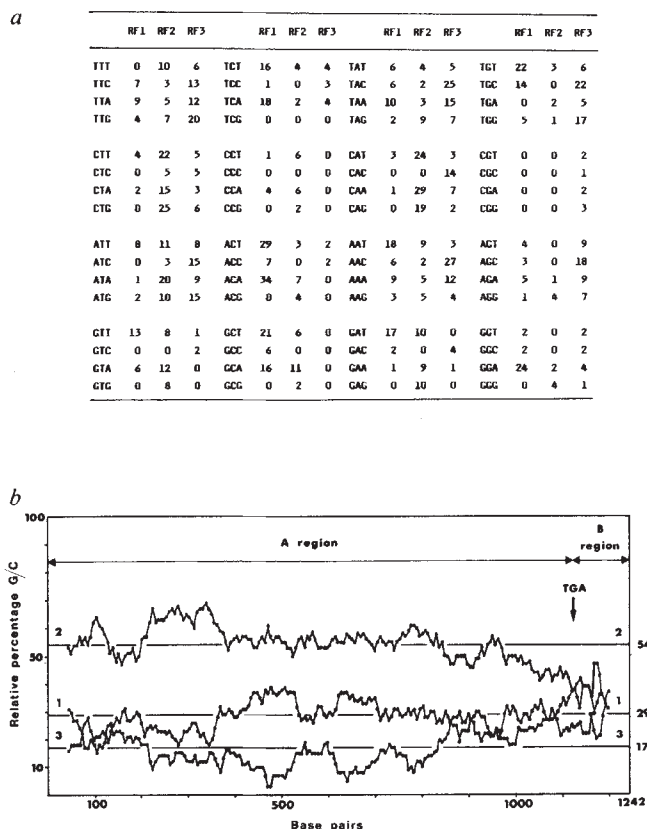


Fig. 2 Codon usage and frequency of G/C bases in the EG2 sequence. **a**, The codon usage in the A region is given for the three possible reading frames RF1, RF2 and RF3. Only RF1 displays a strong bias in favour of A or T at the third position. **b**, The frequency of G/C bases at the three triplet positions. Each point represents the mean value calculated for a 30-triplet fragment (curves 1, 2 and 3 give the relative percentages of G/C at the first, second and third positions, respectively, of the codons in RF1). The three lines are distinct in the A region of the sequence and tend to overlap in the B region. RF1 has the lowest percentage of G/C at the third position.

importantly are characterized by a 3-bp periodicity in A+T richness. As EG2 contains the 3' end of the mRNA, the existence of these two very distinct regions suggests that region A could be the end of the coding sequence. However, the three possible reading frames RF1, RF2 and RF3 all contain scattered TAA, TAG and TGA codons, found every 80 bp on average (see Fig. 1). Yet, if one looks more carefully at the other codons used in the short open reading frames (in between two stop codons) in the A region, one finds that RF1, but not the other two reading frames, shows the following characteristics: (1) The 3-bp periodicity mentioned above falls so that the third base of each codon shows the strongest bias for A and T (see Fig. 2), characteristic of coding sequences in A+T-rich genomes. (2) The ratio of RNY to YNR codons, where R is a purine, N any base and Y a pyrimidine, is equal to 2.5 for the reading frame RF1 and 0.4 and 0.9 for RF2 and RF3 (see Table 1). It has been observed^{12,13} that in many prokaryotic and eukaryotic genomes this ratio is >2 for coding frames and <1 for noncoding frames. (3) The amino-acid composition deduced from RF1 is remarkably close to that of the entire G surface antigen (see Table 1). χ^2 values calculated from the amino-acid composition of G surface antigen¹⁴ are 47 for RF1, 740 for RF2 and 650 for RF3. The same calculation for the last 110 bp of the EG2 sequence (region b), whatever the reading frame, yields higher χ^2 values than for any 110-bp stretch of the A region in RF1 (see Table 1, column i, in which a comparison is made with the first 110 bp of the A region). (4) The nature of the stop codons in RF1 is also peculiar as only TAA and TAG codons are present in region A and a cluster of three TGA codons is located at the junction between A and B (see Fig. 1).

The above arguments, especially those concerning the RNY/YNR ratio, the 3-bp periodicity and the χ^2 values, strongly support the notion that, despite the presence of TAA and TAG triplets in RF1, the A region codes for part of the G antigen.

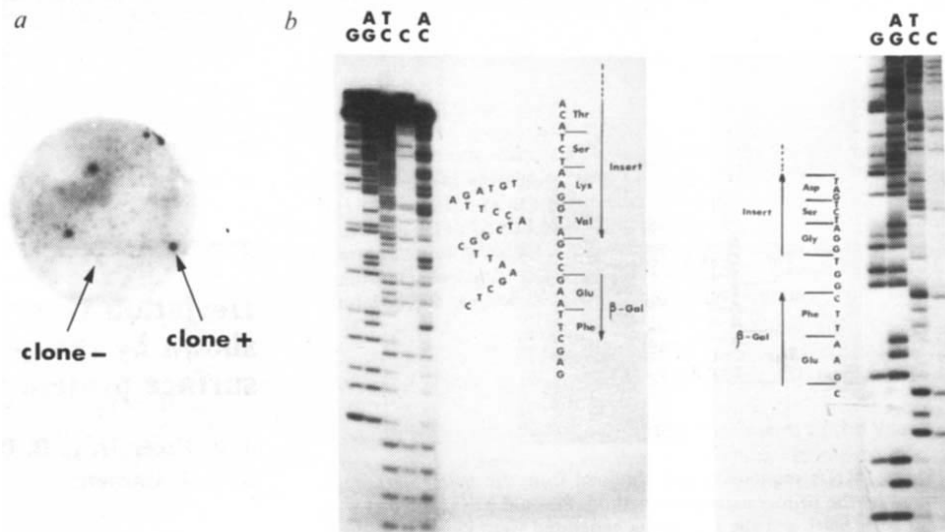
To obtain direct proof that the A region contains coding sequences and that RF1 is the proper reading frame, we used direct expression of DNA subfragments of EG2 in the expression vector λ gt11 to determine whether they code for antigenic determinants which can be recognized by antibodies against G surface antigen. EG2 was cleaved with the restriction enzyme *RsaI* (see Fig. 1), and digested with exonuclease *Bal31* to generate DNA subfragments of various lengths, which were then

Table 1 Amino-acid compositions of A and B regions of EG2 sequence

	a	b	c	d	e	f	g	h	i
Phe	5.8	7	13	19	0.6	6	5	7	1
Leu	17.9	19	79	51	1.8	4	5	1	0
Ser	26.3	42	6	38	2.7	2	1	3	6
Tyr	11.9	12	6	30	1.2	3	1	3	2
Cys	39.5	36	3	28	4.0	0	2	0	4
Trp	4.1	5	1	17	0.4	0	0	0	0
Pro	7.2	5	14	0	0.7	0	0	0	2
His	3.3	3	24	17	0.3	2	0	2	1
Glx	21.5	2	67	10	2.2	2	5	0	0
Arg	5.4	6	5	24	0.5	2	0	1	0
Ile	9.0	9	34	32	0.9	5	8	3	1
Met	1.9	2	10	15	0.2	1	0	1	0
Thr	57.5	70	14	4	5.8	2	1	1	7
Asx	42.2	43	21	34	4.3	4	5	3	5
Lys	21.5	12	10	16	2.2	3	2	5	3
Val	16.4	19	28	3	1.7	0	0	2	1
Ala	47.7	43	19	0	4.8	0	0	0	3
Gly	36.0	28	6	9	3.6	0	1	1	1
TAA		10	3	15		0	2	2	1
TAG		2	9	7		0	0	2	0
TGA		0	2	5		3	0	0	0
Total	375	375	375	375	39	39	39	39	39
χ^2		38	709	583		107	220	112	17
RNY/YNR		2.5	0.4	0.9		0.9	0.8	1.5	2.6

Amino-acid compositions deduced for the A region (columns b, c, d) and the B region (columns f, g, h) of the EG2 sequence in the three reading frames RF1 (b, f), RF2 (c, g) and RF3 (d, h) and for the first 110 bp of the EG2 sequence in RF1 (column i). The amino-acid composition of the G antigen¹⁵ is given for comparison in columns a and e. χ^2 values and the RNY/YNR ratio are also given.

Fig. 3 Determination of the reading frame of the coding sequence of the G surface antigen. 50 µg of plasmid pEG2 (recombinant pBR328 plasmid containing the EG2 sequence at the *EcoRI* site) were digested to completion with the restriction enzyme *RsaI* and then with 1 unit of *Bal31* nuclease (Boehringer) at 37 °C. Aliquots of the reaction mixture were removed after 1, 2, 3, 4, 5, 6, 8 and 10 min of digestion, phenol-extracted and pooled for ethanol precipitation. After repair of the fragments with *E. coli* DNA polymerase, synthetic ³²P-labelled *EcoRI* linkers (P.L. Biochemicals) were ligated to their ends. After digestion with excess *EcoRI*, the DNA fragments (separated from the *EcoRI* linkers by two successive G50 column chromatography steps) were used to produce an expression library in the λ gt11 vector¹⁵ at the *EcoRI* site. The library was screened with antisera-purified antibodies against purified G surface antigen, depleted of any anti-*E. coli* activities. Many positive recombinants were obtained and subcloned twice. **a**, Positive (+) and negative (−) clones from a nitrocellulose replica of phage plaques after incubation with these antibodies, followed by labelling with ¹²⁵I-protein A (Amersham). The insert of one of the positive clones was subcloned in the two possible orientations in the pUC12 vector and its sequence determined by the Maxam and Gilbert technique¹¹ from both ends; it corresponds exactly to the sequence indicated by a dashed line in Fig. 1. Special care was taken to establish the sequence around the two *EcoRI* junctions as it gives the reading frame of the coding sequence of the insert (see text): the two recombinant plasmids bearing the insert in either of the two possible orientations were labelled at the *Bam*HI site, which is contained in the pUC12 polylinker 18 bases from the *EcoRI* site, and were sequenced from the *Bam*HI site towards the *EcoRI* site by the Maxam-Gilbert technique¹¹. The two 20% acrylamide sequencing gels in **b** show that the reading frame of the insert is RF1, as deduced from that of the β-galactosidase.



inserted into the λ gt11 β-galactosidase expression vector¹⁵. Recombinants synthesize hybrid proteins made up of β-galactosidase and part of the polypeptide chain of the G surface antigen, provided the subfragments encode part of the G antigen and are inserted in the correct orientation and reading frame. Furthermore, if the expressed part of the G surface antigen contains an antigenic determinant, the clones synthesizing hybrid proteins can be detected directly by using antibodies against G antigen.

We obtained recombinant clones synthesizing polypeptides that reacted with antibodies against G antigen; one of these clones has been analysed in detail (see Fig. 3). The sequence of the insert is indicated by a dashed line in Fig. 1; this sequence is unique in EG2 and in the entire 15.5-kb fragment. By sequencing the junctions around the *EcoRI* sites, the reading frame of the insert can be deduced from that of β-galactosidase. From the sequences shown in Fig. 3, we conclude that RF1 is the correct reading frame of the G antigen.

Although it is clear that the sequence marked by a dashed line in Fig. 1 contains an antigenic determinant of the G surface antigen and that the open reading frames surrounding this fragment are probably part of the coding sequence as shown above, the punctuation of the coding sequence with TAA and TAG stop codons remains puzzling. One possible explanation is that they are removed by splicing. In order to demonstrate that this is not the case, we sequenced the cytoplasmic mRNA by the primer extension method¹⁶, using as primer the *SphI*/*SphI* fragment included in the fragment expressed in *E. coli* (see Fig. 1). The sequence of the mRNA (Fig. 4) corresponds exactly to the sequence underlined with a solid line in Fig. 1. We conclude from these data that TAA and TAG codons are present in the reading frame of the coding sequence of the G-antigen mRNA.

Thus, TAA and TAG do not represent termination codons in *P. primaurelia*. The simplest explanation of the results is that they code for amino acids; other possible interpretations seem highly unlikely. For example, it could be argued that the gene we have cloned is a pseudogene¹⁷ and that the true gene does not contain TAA and TAG triplets. This interpretation is not plausible for the following reasons: (1) Our S₁ mapping (not

shown) and RNA sequencing experiments indicate that the cloned gene is transcribed abundantly. (2) If the cloned gene is a pseudogene, it must share large segments of homology with the true gene¹⁷. S₁ protection experiments (not shown) performed with EG1, EG2 or subfragments of these have never yielded anything other than a single protected fragment, indicating that the cloned gene and the true gene would differ at most by local changes not revealed by S₁ protection experiments. The 195-bp *SphI*/*SphI* primer, which does not contain TAA and TAG codons and which is, moreover, included in a 336-bp fragment coding for an antigenic determinant of the protein, should also hybridize with the mRNA of the true gene. As the sequence of the cloned gene alone is read and as it is read without ambiguity (see Fig. 4), the true gene should be transcribed at a much lower rate than the cloned gene (that is, at an undetectable level), which is highly unlikely for such an abundant protein. (3) Southern blots of total DNA of *Paramecium* and of the 15.5-kb cloned fragment cut with various restriction enzymes and probed with EG1 or EG2 fragments have always been found to be identical (not shown); this is at variance with the frequent observation that pseudogenes and true genes give rise to different sets of bands on Southern blots¹⁷. (4) The 10 TAA and 2 TAG codons in the EG2 sequence do not seem to be generated by the random mutations expected in a pseudogene. Indeed, despite their presence, the reading frame is unchanged over the whole length of the coding sequence.

Our results lead us to the following conclusions: (1) The fact that TAA and TAG codons are interspersed in the coding sequence (see accompanying paper¹⁸) may explain the blocking of *in vitro* translation of *Paramecium* mRNAs. (2) The localization of the three TGAs in a cluster of four codons just before the end of the transcript, and at the junction between the A and B regions of the gene, suggests strongly that TGA is a termination codon in *Paramecium*. Note that the few genes sequenced to date in ciliates¹⁹⁻²¹ all use TGA termination codons. (3) If TAA and TAG are coding triplets in *Paramecium*, we propose that they code for glutamic acid or glutamine on the basis of their important contributions (30%) to the χ² value. We do not think that many other changes in the *Paramecium* codon assignment

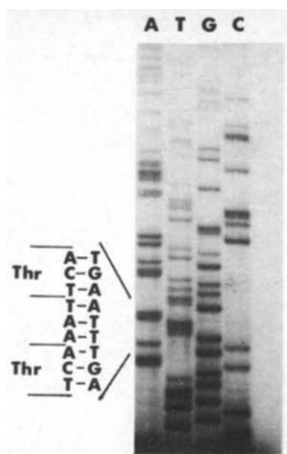


Fig. 4 RNA sequencing gel obtained from the *SphI*/*SphI* fragment by the primer extension method. Plasmid pEG2 (15 µg) was cut with *SphI* and the fragments generated were labelled at their 5' ends with [γ - 32 P]ATP using polynucleotide kinase. The 138-bp long *SphI*/*SphI* fragment contained in EG2 (see Fig. 1) was purified on a polyacrylamide gel; 25% of the recovered probe and 75 µg of total RNA from G-expressing paramecia were ethanol-precipitated and resuspended in 80 µl of 80% formamide hybridization buffer¹⁶. The solution was warmed to 80 °C before being transferred quickly to a 60 °C water bath, the temperature of which was allowed to cool down to 30 °C over 2–3 h. Nucleic acids were precipitated with ethanol and polymerization was performed with reverse transcriptase (Life Science) and a mixture of the four deoxy- and dideoxynucleotides¹⁶. Part of the 6% acrylamide sequencing gel shown gives the RNA sequence around the first TAA codon upstream of the primer.

have occurred as translation of the EG2 sequence according to the universal code leads to a good overall agreement with the amino-acid composition of the G antigen. Direct proof that the TAA and TAG triplets encode amino acids in *Paramecium* has yet to be obtained, and would best comprise a direct comparison of the sequence of a protein with that of its gene, and the demonstration that transfer RNAs bearing anticodons to TAA and TAG triplets exist.

Some evidence that TAA encodes an amino acid in *Tetrahymena*²² and *Stylonichia*²³ has been reported recently; this deviation from the universal code might be peculiar to all ciliates. It would be interesting to know whether it is linked to the existence of nuclear differentiation in ciliates and in particular whether the micronuclear and macronuclear copies of the genes are identical in this respect. This first example of an altered genetic code apart from that in mitochondria may provide new clues as to the evolution of the genetic code.

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Deviation from the universal code shown by the gene for surface protein 51A in *Paramecium*

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The immobilization antigens (i-antigens) of *Paramecium*, large polypeptides of relative molecular mass ~300,000 (ref. 1), are located on the cell surface. Each i-antigen is encoded by a different unlinked gene, and no more than one gene is expressed at a time. The proteins and the mRNAs and genes encoding them are readily isolated^{2,3}. Here we report the nucleotide sequence of three regions of the A i-antigen gene from stock 51 of *Paramecium tetraurelia*. Surprisingly, all reading frames contain TAA and TAG stop codons, even though there is evidence that one reading frame of these sequences codes for the i-antigen. We suggest that in *Paramecium* UAA and UAG code for amino acids, instead of serving as translational stops as they do in all other organisms.

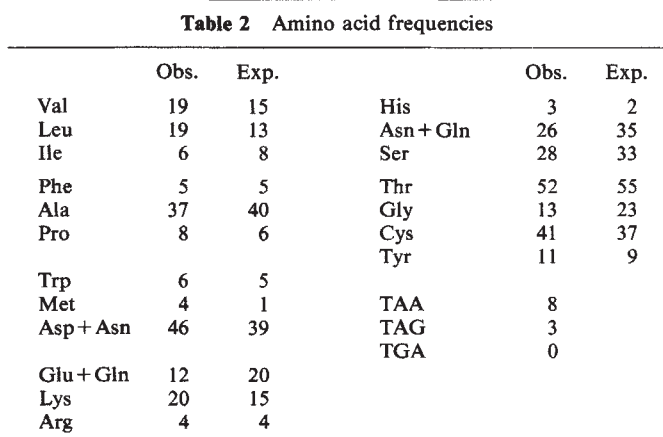
A SA-1 contains the whole transcribed portion of the A gene, ~8.4 kilobases (kb), together with the 5' and 3' flanking regions³. We sequenced 681 bases at the 5' end of the gene and ~400 bases farther downstream (Fig. 1). The start of transcription has been localized previously in an S₁ protection experiment to a point ~450 bases to the left of a *HindIII* site³. In a similar S₁ nuclease experiment, using a shorter fragment and determining more precisely the size of the protected fragment on a sequencing gel (data not shown), the start of transcription was more precisely located 470 bases to the left of the *HindIII* site.

Each of the reading frames subsequent to the first ATG (+15) after the start of transcription (+1) has TAA or TAG stop codons

Table 1 Analysis of reading frames

Region of gene	Reading frame	No. of stop codons			Fit to protein	RNY:YNR
		TAA	TAG	TGA		
-181 to +14	1	3	1	3	182	18:18
	2	5	0	3	102	16:20
	3	5	0	3	108	22:15
+15 to +681	1	4	2	0	26	76:34
	2	5	3	3	503	36:65
	3	5	5	12	404	52:64
+1,126 to +1,291	1	1	0	0	13	21:7
	2	2	0	0	205	4:15
	3	5	1	2	113	13:17
+5,486 to +5,692	1	3	1	0	17	30:8
	2	0	0	0	173	18:22
	3	4	1	5	147	6:24

Each of three possible reading frames is analysed for four regions of the 51A genes. The last three regions are believed to be translated. The number of stop codons, χ^2 fit to overall protein composition and ratio of RNY to YNR codons are given (R is purine, Y is pyrimidine and N is any base).



To explore possible amino acid assignments for TAA and TAG, the frequencies of amino acids predicted by the observed codons for all three translated regions are given in Table 2, together with the numbers expected from the overall amino acid composition of i-antigen A. Ignoring TAA and TAG, the χ^2 for these observed and expected values is 23.0. If TAA and TAG are assigned to specific amino acids, only three of the 20 possible assignments result in lower χ^2 values: Glu ($\chi^2 = 19.9$), Gly (18.8) and Gln (16.5). Therefore, the data show that the best fit is obtained if glutamine is encoded by TAA and TAG. Normal codons for glutamine are CAA and CAG, both of which are found in our sequences. The data of Caron and Meyer⁸ show that the level of Glu + Gln is low. Helftenbein⁹ reports a TAA that appears to specify glutamine in several α -tubulin genes in *Stylonychia*, and Horowitz and Gorovsky¹⁰ have found a TAA specifying glutamine in a histone gene in *Tetrahymena*. Perhaps TAA specifies glutamine in all ciliated protozoa. Finally, the total of 345 codons in reading frame 1 includes 8 TAA, 3 TAG and 0 TGA, suggesting that UGA alone is used as a translational stop by *Paramecium*.

Table 2 Amino acid frequencies

Obs.	Exp.
1	1
2	2
3	3
4	4
5	5
6	6
7	7
8	8
9	9
10	10
11	11
12	12
13	13
14	14
15	15
16	16
17	17
18	18
19	19
20	20
21	21
22	22
23	23
24	24
25	25
26	26
27	27
28	28
29	29
30	30
31	31
32	32
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34	34
35	35
36	36
37	37
38	38
39	39
40	40
41	41
42	42
43	43
44	44
45	45
46	46
47	47
48	48
49	49
50	50
51	51
52	52
53	53
54	54
55	55
56	56
57	57
58	58
59	59
60	60
61	61
62	62
63	63
64	64
65	65
66	66
67	67
68	68
69	69
70	70
71	71
72	72
73	73
74	74
75	75
76	76
77	77
78	78
79	79
80	80
81	81
82	82
83	83
84	84
85	85
86	86
87	87
88	88
89	89
90	90
91	91
92	92
93	93
94	94
95	95
96	96
97	97
98	98
99	99
100	100

Obs.	Exp.
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Val	19	15	His	3	2
Leu	19	13	Asn + Gln	26	35
Ile	6	8	Ser	28	33
Phe	5	5	Thr	52	55
Ala	37	40	Gly	13	23
Pro	8	6	Cys	41	37
			Tyr	11	9
Trp	6	5			
Met	4	1	TAA	8	
Asp + Asn	46	39	TAG	3	
			TGA	0	
Glu + Gln	12	20			
Lys	20	15			
Arg	4	4			

To explore possible amino acid assignments for TAA and TAG, the frequencies of amino acids predicted by the observed codons for all three translated regions are given in Table 2, together with the numbers expected from the overall amino acid composition of i-antigen A. Ignoring TAA and TAG, the χ^2 for these observed and expected values is 23.0. If TAA and TAG are assigned to specific amino acids, only three of the 20 possible assignments result in lower χ^2 values: Glu ($\chi^2 = 19.9$), Gly (18.8) and Gln (16.5). Therefore, the data show that the best fit is obtained if glutamine is encoded by TAA and TAG. Normal codons for glutamine are CAA and CAG, both of which are found in our sequences. The data of Caron and Meyer⁸ show that the level of Glu+Gln is low. Helftenbein⁹ reports a TAA that appears to specify glutamine in several α -tubulin genes in *Stylonychia*, and Horowitz and Gorovsky¹⁰ have found a TAA specifying glutamine in a histone gene in *Tetrahymena*. Perhaps TAA specifies glutamine in all ciliated protozoa. Finally, the total of 345 codons in reading frame 1 includes 8 TAA, 3 TAG and 0 TGA, suggesting that UGA alone is used as a translational stop by *Paramecium*.

(2) It is unlikely that the gene sequenced is a pseudogene or a regulatory gene. Correlations between the size and abundance of the i-antigens and a series of poly(A)⁺ RNAs provide strong evidence that the RNAs code for the i-antigens². The sequences in λ SA-1 hybridize to the RNA judged to be the mRNA for i-antigen A. Genomic Southern transfer experiments indicate that there is probably only one copy of the sequences in the SA-1 genome³. Furthermore, there are deletion mutations that eliminate these sequences and simultaneously eliminate the ability of stock 51 to produce i-antigen A⁴. We have crossed a strain of stock 51 bearing a deletion of the gene found in λ SA-1 with another stock of *P. tetraurelia* (stock 29) bearing the A allele. Alleles 29A and 51A determine i-antigens that can be distinguished serologically¹. Antigen 29A and the deletion were found in the F₂ offspring in a 1:1 ratio, as expected if the deletion included the sequences responsible for serological specificity of

The use of UAA and UAG codons by ciliates should make their mRNAs poor templates for *in vitro* protein synthesis in heterologous systems, indeed numerous workers have found this to be the case^{2,11-13}. Meyer *et al.* have concluded recently that the primary structure of the mRNAs from *Paramecium* is at fault¹⁴.

We conclude that UAA and UAG do not represent stop codons in *Paramecium*; the only other deviations from the 'universal' genetic code have been found in mitochondria.

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Utilization of one promoter by two forms of RNA polymerase from *Bacillus subtilis*

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Bacillus subtilis possesses several forms of RNA polymerase, each differing in its σ subunit and its specificity of promoter recognition^{1,2}. The sequential appearance of σ subunits, which change the promoter recognition specificity of RNA polymerase, may have a key role in controlling the temporal pattern of gene expression required for endospore development in *B. subtilis*³. Several genes that are expressed over relatively long periods of time during the developmental cycle are transcribed by more than one form of RNA polymerase, which initiate transcription from either tandem or overlapping promoters^{2,4}. The promoter region for the *ctc* gene is interesting because transcription is initiated at or near the same position by both σ^{37} RNA polymerase ($E\sigma^{37}$), a minor form in growing cells, and σ^{29} RNA polymerase ($E\sigma^{29}$), a form which appears ~2 h after the initiation of sporulation⁵. Here we report that several base substitutions in the *ctc* promoter differentially affect the utilization of the promoter by $E\sigma^{37}$ or $E\sigma^{29}$.

We have reported previously the use of site-directed bisulphite mutagenesis to construct a series of single and multiple base substitutions in the *ctc* promoter⁶. For this purpose, we cloned a 130-base pair (bp) DNA fragment containing the *ctc* promoter into the *Escherichia coli* phage M13 mp9 to produce phage M13-31 (Fig. 1). After mutagenesis and transfection⁶, the 143-bp *EcoRI*/*HindIII* DNA fragment containing the promoter region was excised from each phage DNA and ligated between the *EcoRI* and *HindIII* restriction sites of plasmid pUC8 (ref. 7). The structure of each plasmid was confirmed by determining the nucleotide sequence of the *EcoRI*/*HindIII* DNA fragment (Table 1).

To test the function of the mutant promoters, we assayed their ability to direct transcription by $E\sigma^{29}$ and $E\sigma^{37}$ in an *in vitro*

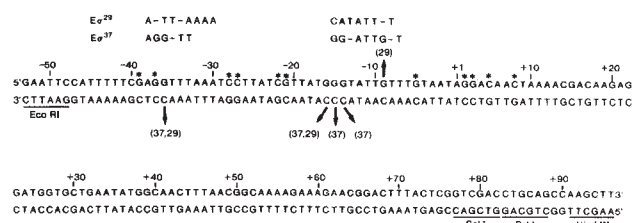


Fig. 1 Structure of the *ctc* promoter region, showing the nucleotide sequence of the *ctc* promoter region which was cloned into M13 mp9 and then recombined into pUC8. Transcription proceeds from left to right, with the lower strand representing the transcribed strand. The start point of transcription is indicated as nucleotide +1. Asterisks represent the positions at which base substitutions (transitions) have little effect on the utilization of this promoter by $E\sigma^{37}$ (ref. 6) or $E\sigma^{29}$. The arrows indicate those positions at which single base transitions more dramatically affect the utilization of the promoter by $E\sigma^{37}$ or $E\sigma^{29}$ as indicated (arrows pointing down indicate a decrease in utilization and the arrow pointing up indicates an increase in utilization). The nucleotide sequences shown above the *ctc* promoter sequence are the sequences (non-transcribed strand shown) which are conserved in promoters recognized by $E\sigma^{37}$ or $E\sigma^{29}$ as indicated^{6,8}.

run-off assay. Template DNA was prepared by cutting the plasmid DNA at the unique *HindIII* restriction site 95 bp downstream from the startpoint of transcription (Fig. 1). $E\sigma^{37}$ or $E\sigma^{29}$ RNA polymerase was incubated with an excess of the cut template DNA and the discrete run-off transcripts visualized by autoradiography after electrophoresis in a polyacrylamide/urea gel. Figure 2 shows the transcripts generated from a series of mutant promoters with single base substitutions at positions occupied by guanine in the non-transcribed strand of the wild-type promoter. As we had shown previously using phage replicative-form DNA as template⁶, the single base pair substitutions causing the greatest reduction in promoter utilization by $E\sigma^{37}$ were at positions -36, -16, -15 and -14 (Fig. 2, lanes d, f, g, h, respectively, and Table 1).

Each of the cut templates was also transcribed by an equal amount of $E\sigma^{29}$ in reactions that otherwise were identical to the $E\sigma^{37}$ transcriptions. Transcription of the wild-type template by $E\sigma^{29}$ results in a 95-base transcript which co-migrates during electrophoresis in a high-resolution nucleotide sequencing gel with the 95-base transcript generated by $E\sigma^{37}$ (K.M.T. and C.P.M. Jr, unpublished data). We also observed some non-specific transcription products that migrate more slowly than the 95-base run-off transcript (Fig. 2, lane k). The intensity of this nonspecific background did not correlate with the intensity of the run-off transcript (Fig. 3, compare lanes f and g).

Transcription of this series of cut templates by $E\sigma^{29}$ revealed that the base substitution at position -15, which greatly reduced transcription by $E\sigma^{37}$, had little effect on transcription by $E\sigma^{29}$ (Fig. 2, lane q). This template was an aliquot of the same cut template used for transcription by $E\sigma^{37}$ (Fig. 2, lane g), thereby providing additional evidence that the inefficiency of transcription of this template by $E\sigma^{37}$ was not caused by the quality of the template preparation (also see mixing experiments in ref. 6). The base substitution at position -9 had little or no effect on transcription by $E\sigma^{37}$, but we observed a slight enhancement of transcription from this template by $E\sigma^{29}$ (Fig. 2, lane s).

Promoters containing single base substitutions at positions -36 or -16 directed less transcription by $E\sigma^{29}$ (Fig. 2, lanes n, p; Table 1). Mixing experiments were used to eliminate the possibility that these template preparations contain an inhibitor of $E\sigma^{29}$. When *PstI*-cut wild-type template was mixed with each *HindIII*-cut mutant template in pre-binding transcription reactions with $E\sigma^{29}$, the mutant templates did not inhibit transcription from the wild-type template (data not shown).

We have also tested promoters containing multiple base substitutions in similar pre-binding transcription reactions (Table

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Fig. 2 Run-off transcripts from mutant promoters. $E\sigma^{37}$ from *B. subtilis* JH646 (*spoOA*) was purified as described elsewhere⁶ and was shown to contain no contaminating $E\sigma^{32}$ or $E\sigma^{29}$ by *in vitro* transcription of plasmids pCB1291 and pMB9, which contain promoters utilized uniquely by $E\sigma^{32}$ and $E\sigma^{29}$, respectively^{2,5}. The results were reproduced with $E\sigma^{37}$ isolated from *B. subtilis* (SMY). $E\sigma^{29}$ was purified on two different occasions from *B. subtilis* (SMY) by gradient elution from DNA-cellulose as described by Haldenwang *et al.*⁵ and shown to be free of contaminating $E\sigma^{32}$ and $E\sigma^{37}$ by transcription of pCB1291, which contains promoters used uniquely by $E\sigma^{37}$ and $E\sigma^{32}$ (ref. 2). We have not excluded the possibility that in addition to their sigma subunits, these holoenzymes differ by modifications that cannot be detected by electrophoresis in SDS-polyacrylamide gels. Addition of a mixture of $E\sigma^{37}$ and $E\sigma^{29}$ to a mutant template has the expected additive effect; therefore, the differential effects are not due to impurities in the polymerase preparations. RNA was transcribed as described previously⁶ by $E\sigma^{37}$ (lanes a-j) or $E\sigma^{29}$ (lanes k-t) from the following templates that had been cleaved at the *Hind*III site 95 bp downstream from the *ctc* promoter: pUC8-31, lanes a, k; pUC8-39, lanes b, l; pUC8-37, lanes c, m; pUC8-36, lanes d, n; pUC8-21, lanes e, o; pUC8-16, lanes f, p; pUC8-15, lanes g, q; pUC8-14, lanes h, r; pUC8-9, lanes i, s; pUC8-5, lanes j, t. The number above each lane refers to the position of the base substitution in the *ctc* promoter region of the template. The asterisks denote the position of the 95-base run-off transcript generated by $E\sigma^{37}$ or $E\sigma^{29}$ as indicated. The transcripts were visualized by autoradiography after electrophoresis in a 7 M urea/9% (w/v) polyacrylamide gel.

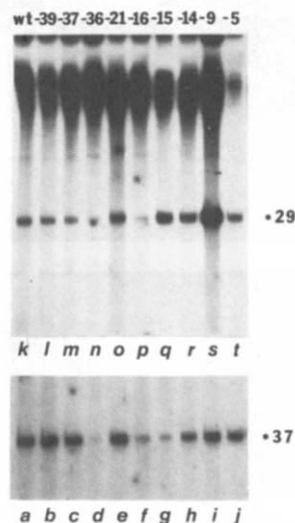
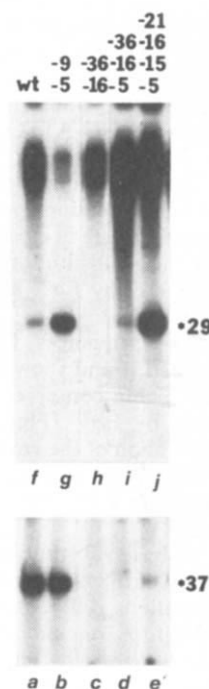


Fig. 3 Run-off transcripts from mutant promoters. RNA was transcribed with $E\sigma^{37}$ (lanes a-e) or $E\sigma^{29}$ (lanes f-j) from the following plasmid templates that had been cleaved at the *Hind*III site 95 bp downstream from the *ctc* promoter: pUC8-31, lanes a, f; pUC8-313, lanes b, g; pUC8-3111, lanes c, h; pUC8-3118, lanes d, i; pUC8-3146, lanes e, j. The number above each lane refers to the positions of the base substitutions in the *ctc* promoter region of the template (see Table 1). The asterisks denote the position of the 95-base run-off transcripts generated by $E\sigma^{37}$ or $E\sigma^{29}$ as indicated. The transcripts were visualized by autoradiography after electrophoresis in a 7 M urea/9% (w/v) polyacrylamide gel.



multiple base substitutions including positions -36 and -16, were used less efficiently than the wild-type *ctc* promoter by both $E\sigma^{37}$ and $E\sigma^{29}$ (Fig. 3, lanes c, d, h, i).

Although $E\sigma^{37}$ and $E\sigma^{29}$ use the *ctc* promoter to initiate transcription at or near the same position^{5,8}, the present results support a model in which these two forms of RNA polymerase utilize the *ctc* promoter by interacting with different nucleotides within the *ctc* promoter region. The most dramatic demonstration that a single base pair within the promoter is critical for utilization by one form of RNA polymerase, but not the other, is the base substitution at position -15. This base substitution severely limits utilization of the promoter by $E\sigma^{37}$, but not $E\sigma^{29}$. Promoter 3146, which has multiple base substitutions, also demonstrates the differential effects of base substitutions on promoter utilization by $E\sigma^{37}$ and $E\sigma^{29}$.

Can we identify those sequences that signal recognition by each type of RNA polymerase? We have proposed that $E\sigma^{37}$

1, Fig. 3). Promoter 3146, containing base substitutions at positions -21, -16, -15 and -5, directed little or no transcription by $E\sigma^{37}$ (Fig. 3, lane e), but directed transcription by $E\sigma^{29}$ more efficiently than did the wild-type *ctc* promoter (Fig. 3, lane j). Promoter 313, with transitions at positions -9 and -5, was also used more efficiently by $E\sigma^{29}$ than the wild-type *ctc* promoter (Fig. 3, lane g). Two promoters (3111 and 3118), containing

Table 1 Activity of mutant *ctc* promoters

Plasmid	Positions of base substitutions (transitions)	Transcription relative to wild-type <i>ctc</i> promoter (%)				Average of 3 expts ($E\sigma^{37}$)
		Expt 1 ($E\sigma^{29}$)	Expt 2 ($E\sigma^{29}$)	Expt 3 ($E\sigma^{29}$)	Average ($E\sigma^{29}$)	
pUC8-31	Wild type	100	100	100	100	100
pUC8-39	-39, +61	76	85	73	78	110
pUC8-37	-37	67	40	31	46	68
pUC8-36	-36	57	16	21	31	18
pUC8-21	-21, +61	150	130	88	120	95
pUC8-16	-16, +65	30	18	57	35	31
pUC8-15	-15	270	76	140	160	16
pUC8-14	-14, +38	160	68	130	120	60
pUC8-9	-9, +51, +65	910	180	330	470	80
pUC8-5	-5	100	61	140	100	120
pUC8-313	-9, -5, +27, +39, +50, +57, +66	900	1,500	690	1,000	170*
pUC8-3146	-21, -16, -15, -5, +20, +23, +65	1,800	690	500	1,300	22*
pUC8-3111	-36, -16, +30, +66	ND	ND	ND	ND	ND
pUC8-3118	-36, -16, -5, +32	19	19	51	30	ND

Activity was determined by densitometric tracing of autoradiographs. ND, not detected.

*Average of two experiments.

recognizes its cognate promoters by interaction with the nucleotides near the -10 region (10 bp upstream from the startpoint of transcription) and the -35 region (35 bp upstream from the startpoint of transcription) of these promoters⁶. The nucleotide sequence GG-ATTG-T and AGG-TT (non-transcribed strand shown) at the -10 region and -35 region, respectively, are conserved among promoters recognized by $E\sigma^{37}$ (see Fig. 1), and methylation protection experiments reveal that these sequences are in close contact with bound $E\sigma^{37}$ (ref. 9). Finally, the base substitutions that severely affect utilization of the *ctc* promoter by $E\sigma^{37}$ alter bases at positions -36 , -16 , -15 and -14 (ref. 6).

The sequences CATATT-T and A-TT-AAAA (non-transcribed strand shown) at the -10 and -35 region, respectively, are highly conserved among the four promoters known to be used by $E\sigma^{29}$ (Fig. 1)⁸. The base substitutions affecting the utilization of the *ctc* promoter by $E\sigma^{29}$ (positions -36 , -16 and -9) do not replace conserved nucleotides, but may act by affecting $E\sigma^{29}$ contacts with neighbouring conserved nucleotides.

A base substitution at position -36 or -16 decreases utilization of the *ctc* promoter by both $E\sigma^{29}$ and $E\sigma^{37}$, whereas other mutations differentially affect utilization of the promoter. We therefore conclude that these two RNA polymerases utilize the *ctc* promoter by interacting with two different but overlapping sets of nucleotides. According to this model, the *ctc* promoter region is a composite of two overlapping promoters, one set of nucleotides signalling recognition by $E\sigma^{37}$ and an intercalating set signalling recognition by $E\sigma^{29}$.

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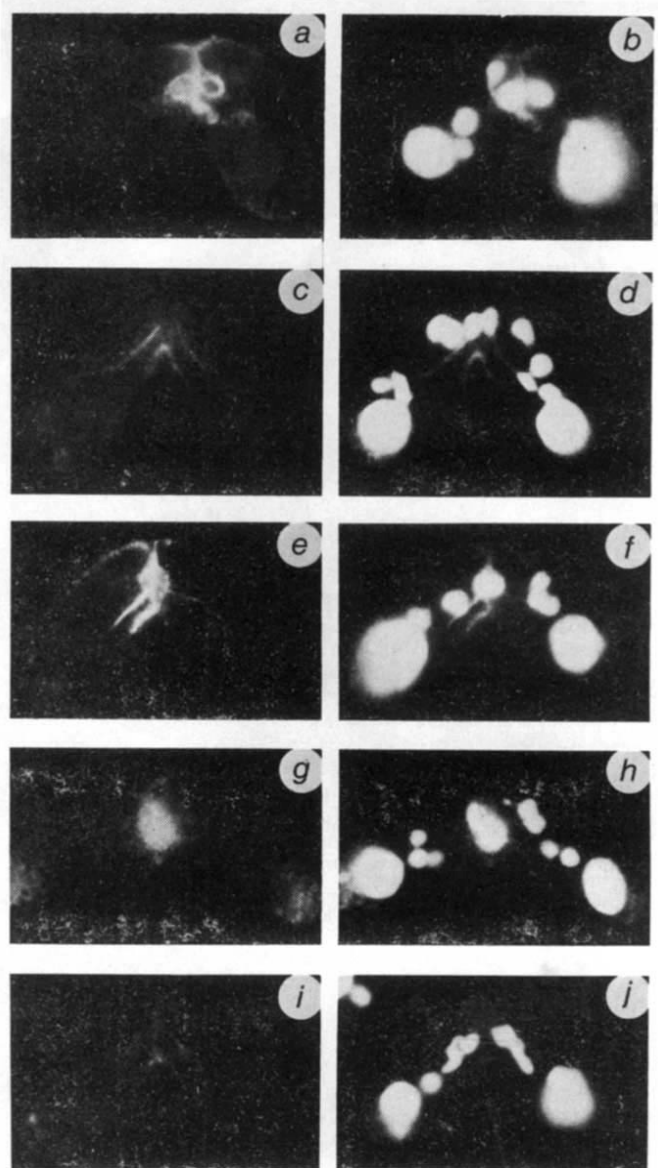


Fig. 1 Immunofluorescence localization of 49K protein in relation to the nuclear events of fertilization in *Tetrahymena*. The conjugation of *Tetrahymena thermophila* (BII and BIII) was synchronized by using a modification of the method of Sugai and Hiwatashi¹⁴. Detergent-extracted cells with conjugating pairs were prepared as described by Goodenough⁶ and air-dried on slides. These unfixed cells were subjected to indirect immunofluorescence staining with anti-49K protein antiserum (diluted 1:160 in phosphate-buffered saline, PBS) and rhodamine-conjugated goat anti-rabbit IgG (diluted 1:200 in PBS; Miles). The macro- and micro-nuclei were stained with $0.5 \mu\text{g ml}^{-1}$ DAPI. *a, c, e, g, i*, Fluorescence photomicrographs of extracted cells stained with anti-49K protein antiserum. *b, d, f, h, j*, Extracted cells stained with anti-49K protein antiserum and DAPI (double exposure). *a* and *b* were taken at 5.75 h, *c* and *d* at 6.5 h, *e* and *f* at 7.0 h, *g* and *h* at 7.25 h, and *i* and *j* at 7.5 h after mixing of complementary mating-type cells. For an explanation of the dotted fluorescence in the cortical regions, see ref. 2.

By using immunofluorescence, we localized the 49K protein in *Tetrahymena* cells at various stages of synchronous conjugation, using an antiserum against 49K protein. Immune blotting experiments have shown that the antiserum is highly specific for 49K protein². Using the monospecific antiserum, we observed that after pair formation in *Tetrahymena*, immunofluorescence appeared in some filamentous structures and/or in an eyeglass-like structure around the cell-cell junction. We were interested

Control of germ cell nuclear behaviour at fertilization by *Tetrahymena* intermediate filament protein

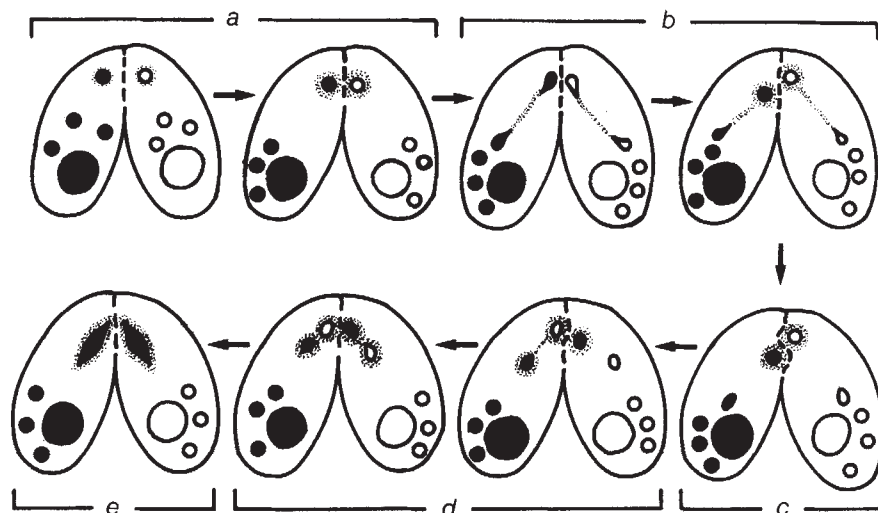
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Intermediate filament protein [relative molecular mass (M_r) 49,000 (49K)] from the ciliated protozoan *Tetrahymena* has been shown to resemble intermediate filament proteins from mammalian cells in several respects¹, and to have a possible role in the oral morphogenesis preceding binary fission in *Tetrahymena*². Here, based on immunofluorescence localization of the 49K protein in *Tetrahymena* during the early stages of conjugation, we suggest that the protein is involved in some nuclear events³⁻⁵, such as the production of four haploid nuclei by prezygotic divisions (meiosis), selection of one of the four meiotic products, formation of the gametic pronucleus by the mitotic division of the selected meiotic product, transfer of the gametic pronucleus across a cell-cell junction, and zygote formation by pronuclear fusion.

Fig. 2 Schematic diagram of the change in the distribution of 49K protein at conjugation in *Tetrahymena*: *a-e* represent the stages of conjugation. *a*, Only one of four meiotic products approaches the junction of the two cells (see Fig. 1*a, b*). *b*, This product divides mitotically to yield the migratory and stationary pronuclei (see Fig. 1*c-f*). *c*, The migratory pronucleus moves across the cell junction (refer to Fig. 1*g, h*). *d*, The stationary pronucleus moves towards the incoming migratory pronucleus (Fig. 1*i, j*). *e*, Two gametic pronuclei fuse to generate a fertilization nucleus. The fine black dots show the localization of the 49K protein.



in these fluorescence patterns because they seemed to reflect the behaviour of germ cell nuclei at conjugation. Because the behaviour of the nucleus can be considered as a form of cell motility and since the 49K protein is thought to constitute the cytoskeleton responsible for cell motility, immunofluorescence staining was performed on detergent-extracted cells prepared by the method of Goodenough⁶. The fluorescence patterns of these extracted cells, taken from various stages of conjugation, were essentially the same as those of whole cells, a finding that suggests strongly the importance of cytoskeletal 49K protein. Furthermore, cytoskeletal 49K protein and germ cell nuclei are located close to each other, as revealed by extracted cells double-stained with rhodamine-conjugated antibody and DAPI(4',6-diamidino-2-phenylindole dihydrochloride) (see Fig. 1).

Next, we determined the sequence of the stages showing the various immunofluorescence patterns by measuring the frequency of occurrence of each stage (data not shown); fluorescence in the functional oral apparatus disappeared 2 h after mixing of the complementary mating-type cells. The first and second prezygotic divisions (meiosis) occurred ~4 h and 4.5 h, respectively, after mixing, and resulted in the production of four haploid nuclei. At a later stage of the second prezygotic division, filamentous structures linking the separating nuclei showed transitory fluorescence (results to be published elsewhere). At 5.75 h after mixing, one of the four meiotic products had approached the junction of the two cells; the periphery of this product fluoresced strongly, whereas the peripheries of the other three products showed no fluorescence (Fig. 1*a, b*). The former is known to become the functional germ nucleus and the latter three the relic nuclei. At 6.5 h, only the functionally active nucleus divided mitotically to give rise to the migratory and stationary gametic pronuclei. At the separation stage of mitosis, a filamentous structure linking the two pronuclei fluoresced conspicuously (Fig. 1*c, d*); immediately after this, the periphery of the presumptive migratory pronucleus fluoresced again and the filament between the pronuclei began to show diminished fluorescence (Fig. 1*e, f*). At 7.0 h after mixing, the migratory pronucleus was about to move across the cell junction; intense fluorescence was observed around the migratory pronucleus, whereas fluorescence from the filamentous structure disappeared (Fig. 1*g, h*). At 7.25 h, corresponding to the stage just after pronuclear exchange but before pronuclear fusion, a filamentous structure linking the incoming migratory nucleus and the stationary nucleus fluoresced again (Fig. 1*i, j*) and the peripheries of the gametic pronuclei also fluoresced. At this stage, we found a remarkable difference between the stationary pronucleus and the relic nuclei in terms of immunofluorescence. The filamentous structure shortened as the pre-fusion process

progressed. At 7.5 h after mixing, the gametic pronuclei fused to form the fertilization nucleus.

It has long been thought that in *Tetrahymena* and *Paramecium*, the determination, differentiation and behaviour of germ nuclei during conjugation result from localized differences in the cytoplasm surrounding them^{3,7-10}, but there is no evidence of such cytoplasmic controlling factors. The present study provides some important clues for solving this problem: *Tetrahymena* intermediate filament protein (49K protein) is presumably involved in the germ nuclear events (see Fig. 2, which summarizes the results of the present experiments schematically). From the immunofluorescence localization of 49K protein, we believe that the protein is involved to some extent in: (1) the selection of one functional nucleus from four haploid nuclei generated by meiosis (Fig. 2*a*); (2) the division of the functional nucleus to generate migratory and stationary pronuclei (Fig. 2*b*); (3) the determination of the migratory pronucleus (Fig. 2*b*); (4) the migratory pronuclear exchange (Fig. 2*c*); (5) the selection of two gametic pronuclei from five nuclei (including three relic nuclei) to form the fertilization nucleus (Fig. 2*d*); and (6) the fusion of gametic pronuclei (Fig. 2*d, e*).

It is known that gametic pronuclear exchange is blocked by inhibitors of microtubule assembly¹¹. Recently, Orias *et al.*¹² showed that each migratory pronucleus is surrounded by a microtubular meshwork. We speculate that, as well as the microtubular meshwork, the cytoskeletal 49K protein has a crucial role in the transfer of gametic pronuclei across the cell junction, and that 49K protein interacts with the microtubules. As the microtubules are observed at the time of separation of the germ pronuclei (T. Yasuda, personal communication), the 49K protein may also be present owing to its association with the microtubules. Such an association does not, however, hold true for all the cases in which microtubules are present in large numbers: ciliary axonemes, most parts of the oral apparatus, cortical microtubular bands, and the micronucleus at the crescent stage of meiotic prophase¹³ showed no anti-49K protein immunofluorescence. Thus, we conclude that *Tetrahymena* intermediate filament protein may have crucial roles in fertilization either alone or in association with some microtubules having specialized functions.

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Reversible cyclic AMP-dependent change in distribution of myosin thick filaments in *Dictyostelium*

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Myosin is thought to act as a major mechanochemical transducer in non-muscle cell motility¹, but the *in situ* organization of the molecules has not yet been determined. Here we report the localization of myosin 'rods', analogous to the thick filaments of muscle, by ameliorated immunofluorescence and demonstrate the dynamic translocation of these rods in response to exogenously added cyclic AMP, which is a chemoattractant for *Dictyostelium* amoebae. On addition of cyclic AMP, we observed instantaneous shedding of the endoplasmic myosin followed by an increase in cortical rods, the original distribution being recovered in a few minutes. We conclude that myosin filaments mediate *Dictyostelium* cell movement, probably by an assembly/disassembly cycle of the molecules in response to a chemotactic stimulus.

We established a hybridoma that produces a monoclonal antibody (IgG) against *Dictyostelium* myosin. This antibody recognizes myosin heavy chain, using immunoblotting techniques². Immunogold staining of purified *Dictyostelium* myosin shows that the antibody is reactive to reconstituted myosin thick filaments (Fig. 1a, b). We have modified our 'agar-overlay' immunofluorescence technique, which enables us to localize *Dictyostelium* actin, myosin and tubulin^{2,3}. Our improved fixation protocol allows the specific visualization of fluorescent rod-like shapes (Fig. 1c). The rods are uniform in size (0.1-0.2 µm wide, 0.7 µm long) and identical to those found with immunofluorescent staining of reconstituted thick filaments of *Dictyostelium* myosin (Fig. 1d). The presumptive myosin rods are localized at the cortical region of vegetative cells, shown by fluorescence micrographs taken at several focal planes (Fig. 2a-d).

We believe that our fixation procedure reflects the *in situ* form of the molecules as: (1) the samples were fixed instantaneously in cold methanol-containing formalin and (2) once fixed, the rods did not dissolve even when the phosphate-buffered saline (PBS) was replaced with myosin solubilizing solution (containing 0.6 M KCl). We found no thick filaments in transmission electron micrographs of thin sections of cells prepared for the immunofluorescence experiments. We next investigated the possible disruption of the thick filaments by our fixation method (Table 1). Fixation with 0.1% OsO₄ for 10 min at 4 °C destroyed the rod-like structures, leaving only weak dot-like fluorescence. Thus, non-muscle myosin thick filaments are susceptible to chemical fixation, which may be the reason for the controversy about their existence in non-muscle cells^{4,5}.

A vegetative cell undergoes cytokinesis every 3 h. At this stage, the cortical fluorescence mostly disappears except in part of the cleavage furrow. The myosin rods are aligned in parallel with each other, forming a circular and peripheral ring-shaped fluorescence (Fig. 2e-h). This suggests the presence of a contractile ring⁶ of microfilaments at the constricting portion of the dividing cells.

When the amoebae start development, their motile activity increases. During the initial 2 h of development, myosin rods increase in number and appear in the endoplasm side by side in the cortex (Figs 2k; 3a, c). In polarized cells undergoing

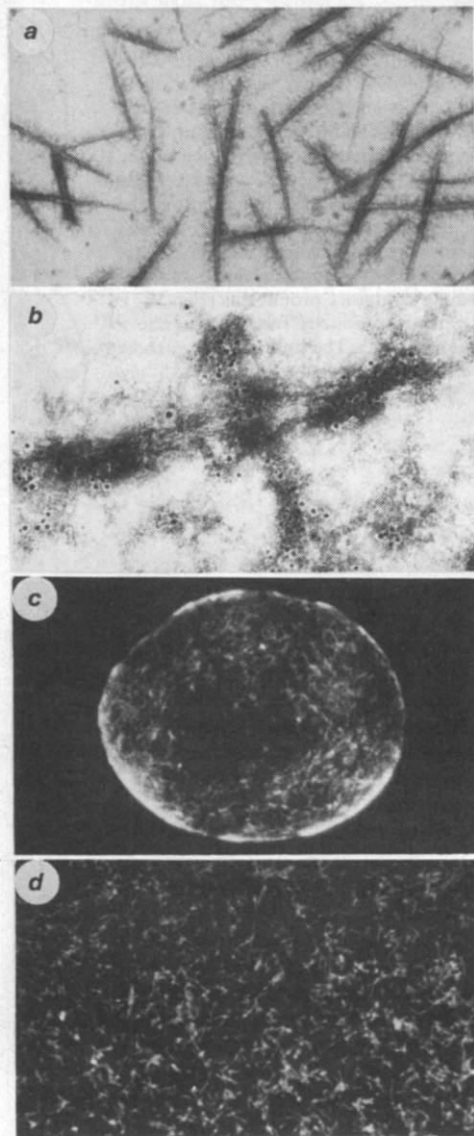


Fig. 1 Electron and immunofluorescence micrographs of reconstituted thick filaments of *Dictyostelium* myosin and *in situ* localization of presumptive thick filaments by agar-overlay immunofluorescence². *a*, Negative staining of reconstituted thick filaments, $\times 29,600$. Assembled myosin molecules form spindle-shaped bipolar filaments. *b*, Immunogold staining of purified myosin filaments, $\times 118,400$. *c*, Immunofluorescence micrograph of a *Dictyostelium* cell prepared by agar-overlay immunofluorescence, $\times 1,739$. *d*, Immunofluorescent staining of purified myosin thick filaments, $\times 1,739$.

Methods. *Dictyostelium* myosin was purified by the method of Mockrin and Spudich¹⁵ and the thick filaments formed by dialysis against a low salt buffer (50 mM KCl, 1 mM EDTA, 10 mM MgCl₂, 1 mM dithiothreitol (DTT), 10 mM imidazole, pH 6.5). *b*, Reconstituted thick filaments were placed on a poly-L-lysine-coated grid, incubated with our myosin antibody and stained with colloidal gold-conjugated second antibody (GAM-G5, Janssen). The monoclonal anti-*Dictyostelium* myosin binds to the thick filaments. *c*, Cells were placed on a coverslip, overlaid with a thin agarose sheet and immediately immersed into methanol containing 1% formalin at -15 °C, then fixed for 5 min. After washing with PBS, the cells were incubated with the anti-*Dictyostelium* myosin for 30 min and then fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG (Miles-Yeda) for 30 min at 37 °C. *d*, Reconstituted thick filaments were placed on a poly-L-lysine-coated coverslip, incubated with our antibody and stained with FITC-labelled second antibody.

Fig. 2 Specific distribution of myosin rods in *Dictyostelium discoideum* amoebae shown by agar-overlay immunofluorescence². *a-d*, Phase-contrast (*a*) and fluorescence (*b-d*) micrographs showing specific localization of myosin rods at the cortical region of a vegetative cell. $\times 1,572$. Immunofluorescence micrographs taken at three different focal planes of the cell verified that the rods were localized only at the upper (*b*) and lower (*d*) cortices. Note that the rods are aligned in parallel with the cortex. *e-h*, Phase-contrast (*e*) and fluorescence (*f-h*) micrographs showing the distribution of myosin rods aligned in parallel forming a contractile-ring-like array. $\times 1,443$. *f-h*, Immunofluorescence micrographs taken at three different focal planes of the cell performing cytokinesis. The micrographs taken at the upper (*f*), middle (*g*), or lower (*h*) plane confirmed that the rods were arranged in a peripheral circular ring at the cleavage furrow. The number of rods at the cell cortex other than the constricting portion have decreased. *i-k*, Phase-contrast (*i*) and immunofluorescence (*j, k*) micrographs showing that the myosin rods are localized specifically in the posterior cortex of a polarized cell and form linear alignments. *j, k*, Immunofluorescence micrographs taken at the upper (*j*) or middle (*k*) plane demonstrate that the rods are localized in the endoplasm (*k*) as well as in the cortex (*j*) at this stage. $\times 1,572$.

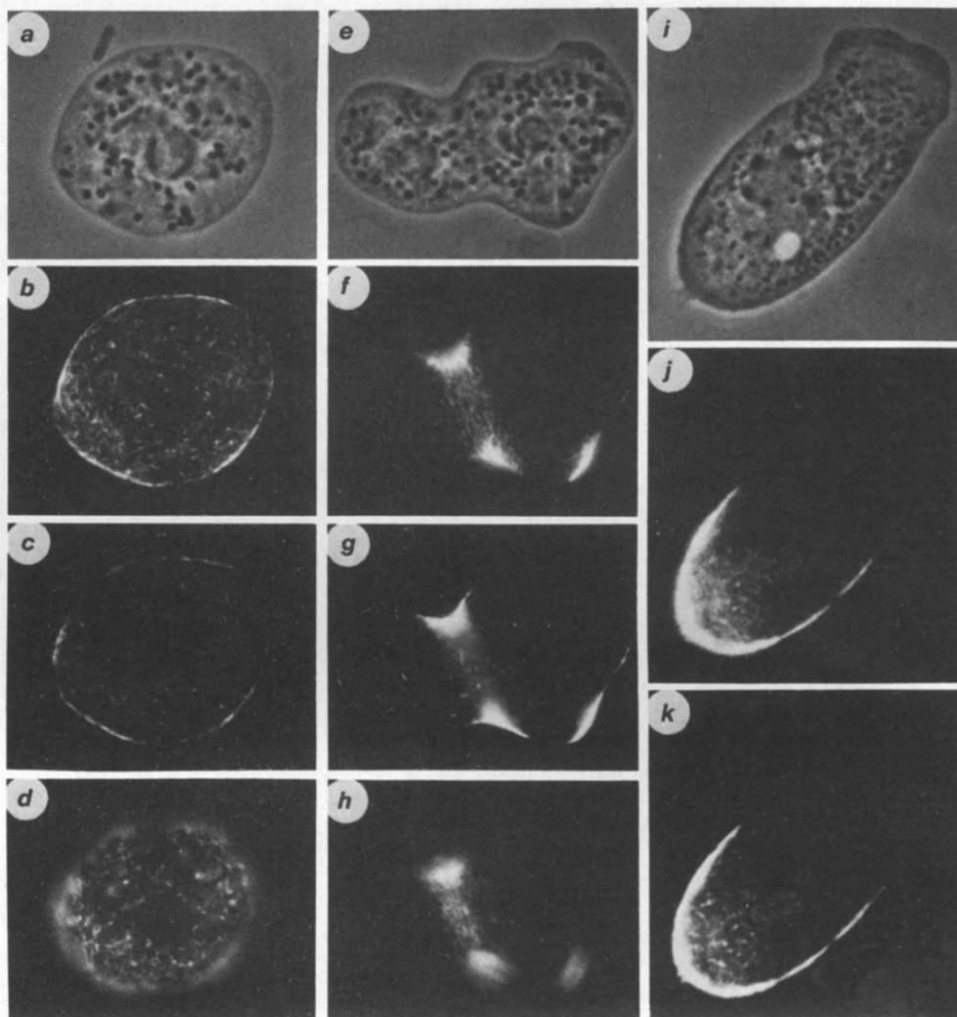


Table 1 Effects of chemical fixation on the disruption of *in situ* myosin rods of *Dictyostelium*

Glutaraldehyde %	OsO ₄ %	Myosin rods
0	0	+
1	0	?
2	0	—
0	0.01	+
0	0.1	??
1	0.01	—
2	0.1	—

A stock solution of glutaraldehyde (25%; Electron Microscopy Science) was diluted with PBS (138 mM NaCl, 2.7 mM KCl, 8 mM phosphate buffer, pH 7.2). The sample was fixed for 30 min at 25 °C. Stock solution of OsO₄ (8%; Merck) was diluted with PBS and the sample fixed for 10 min at 4 °C. The degree of fluorescent staining of myosin rods was judged as follows: —, no specific rod-like fluorescence detectable, partially because of autofluorescence from glutaraldehyde; +, normal rod-like fluorescence; ?, faint rod-like fluorescence identified against the bright autofluorescence; ??, fluorescent dots were seen instead of rod-like shapes.

directed locomotion with the anterior pseudopod, we find that the rods accumulate in the posterior cortex. Occasionally, some linear alignments of the rods occur just beneath the cortex (Fig. 2*i-k*). This evidence supports the suggestion that the force for amoeboid movement is generated by contraction of the posterior cortex^{2,7}.

The assembly of *Dictyostelium* myosin is thought to be regulated by heavy-chain phosphorylation; dephosphorylation

would cause the monomeric myosin to form thick filaments with much actin-activated ATPase⁸, in contrast to the situation in mammalian myosins^{9,10}. Malchow *et al.*¹¹ found that the addition of the chemoattractant cyclic AMP to the aggregation-competent cells induced the rapid and transient phosphorylation/dephosphorylation cycle of myosin heavy chains. Do thick filaments perform cyclic changes of disassembly/assembly in response to the cyclic AMP signal? Here, we find that the myosin rods disappear from the endoplasm and accumulate in the ectoplasm within 2 min of treatment with 10⁻⁶ M cyclic AMP at 5 °C. After 1 min, the rods have redistributed throughout the endoplasm (Fig. 3*a-c*). This change in distribution is dramatic and occurs in almost all of the observed cells within 1 min after the addition of cyclic AMP at 11 °C; it is too fast to follow at 23 °C.

If cyclic AMP is applied locally, the cell extends a pseudopod in the direction of the signal within several seconds¹². Our results described here suggest that translocation of the endoplasmic myosin rods is coincident with this response to a chemotactic stimulus. As the accumulation of rods in the cell cortex is transient, we asked whether a cell without these endoplasmic rods could contract. We prepared Triton-insoluble ghosts of cells with endoplasmic rods depleted by treatment with cyclic AMP and examined their contractility in response to ATP. SDS-gel electrophoresis shows that the ghosts are composed mainly of actin and myosin, comprising up to 25% and 50% of the total cellular proteins (Fig. 3*f*). When 1 mM ATP is added to the ghosts, the cortex contracts in a few seconds (Fig. 3*d, e*). This suggests that the cortical myosin rods are organized in such a way to generate the force required for amoeboid movement.

The myosin rods described here are much larger than found normally in non-muscle cells⁴. We believe that the rod-shaped

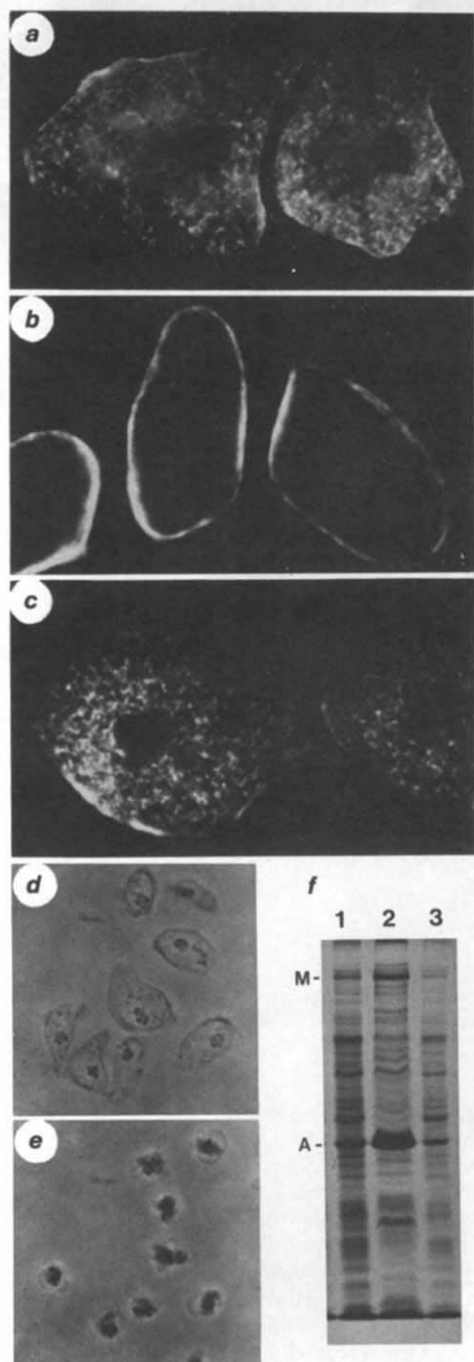


Fig. 3 Changes in the distribution of myosin rods in response to exogenously added cyclic AMP and the contractility of the cell cortex. **A** 10^{-6} M cyclic AMP solution in PBS buffer (15 mM, pH 6.5) was added to aggregation-competent cells overlaid with a thin agarose sheet at 5 °C. After the indicated time, the cells were plunged into -15°C methanol containing 1% formalin and prepared for the agar-overlay immunofluorescence². **a**, Control cell; **b**, 2 min; **c**, 3 min after cyclic AMP application $\times 1,350$. Note the disappearance of the endoplasmic rods and their accumulation at the cortex in **b** and their reappearance in **c**. The same batch of cells was overlaid with a thin agarose sheet and treated with 10^{-6} M cyclic AMP at 5 °C. After 2 min, when the endoplasmic rods disappeared as shown in **b**, the cells were permeabilized with P buffer (10 mM PIPES, 50 mM KCl, 1 mM MgCl_2 , 10 mM EGTA, 1 mM DTT, 0.2 mM phenylmethylsulphonyl fluoride, pH 7.6) containing 0.5% Triton X-100 for 10 min at 0 °C, then extracted with P buffer for 5 min. Phase-contrast micrograph (**d**) shows that the Triton-insoluble ghosts contain the cell cortex, nucleus and some cytoplasmic debris. **d**, **e**, Contracted ghosts a few seconds after application of P buffer containing 1 mM ATP. $\times 417$. For SDS-polyacrylamide gel electrophoresis, a suspension of aggregation-competent cells was treated with 10^{-6} M cyclic AMP for 2 min at 5 °C, then permeabilized with P buffer containing 0.5% Triton X-100 for 10 min on ice. After 5 min centrifugation at 10,000 r.p.m. the pellet and supernatant were separated and boiled for a few minutes in SDS sample buffer (0.25 M Tris-HCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, pH 6.8). The whole-cell lysate was prepared by solubilizing the cells with pre-warmed SDS sample buffer. SDS-polyacrylamide gel electrophoresis was performed¹⁶ and protein quantity was estimated using a Gelman Model DCD-16 densitometer. **f**, Lane 1, whole-cell lysate (100 μg); lane 2, Triton-insoluble proteins (85 μg); lane 3, Triton-soluble proteins (50 μg); **A**, actin; **M**, myosin heavy chain.

fluorescence represents *in situ* myosin thick filaments, partly because of their immunological and morphological characteristics, and partly because of their specific localization in the cleavage furrow, their linear alignment and the dynamic cycle of the possible disassembly/assembly in response to cyclic AMP. The rods observed by immunofluorescence seem larger than

those observed by negative stain (Fig. 1a, d). The size is probably amplified both by the antibody binding and by the fluorescence reflecting fuzzy, unwound projections of the molecules radiating from the rods.

An average cell should contain $\sim 3.8 \times 10^{-10}$ mg myosin, assuming that each cell has 7.5×10^{-8} mg protein and that myosin is 0.5% of the total cell protein¹³, equivalent to 5×10^5 myosin molecules. Assuming that $\sim 75\%$ of the molecules formed thick filaments (suggested by *in vitro* phosphorylation⁸) and that each thick filament is composed of 30 molecules, one cell should contain $\sim 1.3 \times 10^4$ thick filaments. This value is in agreement with the numbers counted here (Fig. 1c). We speculate that these rods are transferred by changes in the molecular assembly of myosin, possibly regulated by the phosphorylation/dephosphorylation of the molecules coupled with the chemotactic response. It is reasonable to suppose that the return of cortical rods to the endoplasm results from ATP-dependent contraction of cortical actomyosin. This idea is supported by the fact that *Dictyostelium* myosin bound to the membrane preparation is released from the cell cortex on treatment with Mg-ATP^{14} .

We conclude that *Dictyostelium* myosin forms thick filaments *in situ* and that translocation of these filaments occurs in coordination with the assembly/disassembly of the molecules in response to physiological changes.

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Association of histone H1⁰ with a gene repressed during liver development

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Histone H1⁰ has a number of unusual properties that set it apart from other H1 subtypes (for review see ref. 1). For example, H1⁰ synthesis is not strictly coupled to DNA synthesis², it is absent from the embryonic liver of mice (but present shortly after birth)^{3,4} and its synthesis is hormone-dependent in some of the glands of adult rodents³. All the H1 subtypes differ in their DNA binding properties, and H1⁰ has been shown to be preferentially associated with nuclease-resistant chromatin⁵. These features suggest that the H1⁰ may have a role in developmental gene control. To investigate this further, we have fractionated the H1⁰-containing nucleosomes of chromatin from adult mouse liver. We report here that the gene for α -fetoprotein, which is expressed in embryonic tissue but repressed soon after birth⁶⁻⁹, is preferentially associated with the H1⁰-containing nucleosomes. The related gene for albumin, which is expressed in both embryonic and adult tissues, is absent from the H1⁰-containing nucleosome fraction. These results support a role for histone H1⁰ in the control of gene expression.

Varshavsky¹⁰ and Smith and Johns¹¹ have demonstrated that nucleosomes can be separated as a function of their H1 content by a non-denaturing polyacrylamide gel electrophoresis system¹². We have adapted this technique for the preparation of H1⁰-containing nucleosomes using a digest of adult mouse liver nuclei in which the amount of H1⁰ relative to total H1 is 35%. As shown in Fig. 1, monomers representing 50% of the total DNA in the conditions of digestion can be resolved into four bands. To characterize the monomers further, the bands were

excised from the gel and their protein content and DNA length analysed. The results are shown in Fig. 1 and summarized in Table 1.

Class 1 and 2 monomers contain no histone H1; class 3 monomers contain histone H1⁰ only, with a slight contamination by H1-2 (<1%), while class 4 monomers contain the full complement of histone H1 (H1-1, H1-2, H1⁰). The amount of H1⁰ relative to total H1 is 20%, indicating that class 4 monomers contain ~20% of nucleosomes bearing H1⁰ if one assumes one molecule of H1 per nucleosome.

The DNA length measurements show that class 1 monomers are actually core particles, while class 2, 3 and 4 monomers have the DNA length expected for nucleosomes, 166–204 base pairs. DNA from the various monomer fractions was purified as described in Fig. 1 legend and spotted in equal amounts on nitrocellulose filters. The filters were hybridized either with a probe for albumin gene (ALB) or with a probe for α -fetoprotein gene (AFP), that is, genes that are, respectively, expressed and not expressed in adult mouse liver.

The DNA of the nucleosome fraction containing only H1⁰ (class 3 monomers) hybridizes strongly with the AFP probe but very weakly with the ALB probe (Fig. 2). In contrast, the DNA of class 1 and 4 monomers hybridizes strongly with the ALB probe but very weakly with the AFP probe (Fig. 2). The DNA purified from unfractionated nucleosomes hybridizes roughly equally with both probes. The fractionation procedure used in our study produces two populations of H1⁰-containing nucleosomes that may reflect heterogeneity in H1⁰ (ref. 13). The 20% of H1⁰-containing nucleosomes in class 4 monomers do not hybridize to the AFP probe, which suggests that their functional properties differ from those of the H1⁰-containing nucleosomes of class 3 monomers.

The present study shows that: (1) A coding sequence expressed in adult liver (ALB) is found in the fraction of class 4 monomers which contains the full complement of H1 histones. The results concerning the presence of H1 in active chromatin are still unclear, but our observation agrees with Berent and Sevall¹⁴ and with Weintraub¹⁵, who have shown that H1 is present in active chromatin. (2) Most of a coding sequence not expressed in the adult liver (AFP) is found in the fraction of monomers containing only H1⁰, while an expressed coding sequence (ALB) is absent from this fraction but found in the

Fig. 1 DNP particle (deoxyribonucleoprotein particle) gel electrophoresis. Adult mouse liver nuclei, prepared as described elsewhere¹⁶, were digested with micrococcal nuclease (200 U nuclease per 10⁸ nuclei, 10 min at 37 °C). The digested nuclei were lysed in 0.2 mM EDTA pH 7, containing 1 mM phenylmethylsulphonyl fluoride. The lysate (10 A₂₆₀ units) was fractionated by electrophoresis on a preparative 5% polyacrylamide slab gel (15 × 30 × 0.3 cm) as described by Varshavsky *et al.*¹². Electrophoresis was performed at 200 V for 16 h at 4 °C with a continuous circulation of the buffer between the two reservoirs. The bands were visualized after ethidium bromide staining (see text for the description of the bands).



The bands were visualized after ethidium bromide staining (see text for the description of the bands). Protein content of the various nucleosomes: The protein content of the DNP gel slice was obtained after electroelution in 0.025 M Tris, 0.192 M glycine, 0.1% SDS pH 8.3 followed by dialysis against water and lyophilization. They were then analysed on an SDS–15% polyacrylamide gel¹⁷ and stained with Coomassie blue. DNA length of the various nucleosomes: The gel slices from the DNP gel were cut into fine particles and incubated overnight at 20 °C in 5 vol. 0.5% SDS, 3 mM EDTA pH 7.6 (ref. 12). Pieces of the gel were then removed by centrifugation at 10,000g for 10 min followed by filtration on glass wool. The supernatant was treated first with 50 g ml⁻¹ RNase T1, 1 h at 37 °C, then with 100 g ml⁻¹ proteinase K, 48 h at 37 °C. The extract was made 1.5 M in NaCl, cooled to 4 °C and SDS was removed by centrifugation at 10,000g for 10 min. The supernatant was extracted with phenol, then with chloroform¹⁸. Chloroform was removed by ether extraction. The DNA was precipitated with 2 vol. ethanol for 24 h at –20 °C in the presence of 10 mM MgCl₂ and recovered after centrifugation at 110,000g for 3 h in a SW 28 rotor. The pellet was then washed with 95% ethanol and DNA reprecipitated as above. Analysis of the DNA length was performed by electrophoresis on a 6% polyacrylamide gel¹⁹. A *Hae*III digest of RFΦX174 was used as size marker. DNA lengths are expressed in base pairs (bp).

Table 1 H1 content and DNA length of the various monomers

Class of monomers	% Monomer (relative proportion)	H1 content	% H1 ⁰ /total H1	DNA length in base pairs at	
				Mean	Halfwidth
1	35	No	—	148 ± 3	140–166
2	10	No	—	183 ± 3	169–202
3	15	H1 ⁰	99	177 ± 3	166–200
4	40	H1-1, H1-2, H1 ⁰	20	180 ± 3	167–204
Native chromatin		H1-1, H1-2, H1 ⁰	35	—	—

H1⁰/total H1 was determined by densitometry after scanning the protein electrophoresis gel (Fig. 1). DNA length, obtained by densitometry scanning of the DNA electrophoresis gel (Fig. 1), is expressed in base pairs at the maximum absorbance and at the halfwidth of the peak.

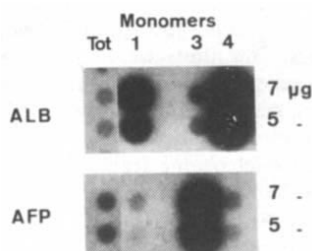


Fig. 2 Albumin and α -fetoprotein sequences among the various monomers. Purified DNAs isolated from the different monomers were hybridized to AFP1 and pmalb2 probes.

Methods. Plasmids were amplified in *Escherichia coli* (HB101) in rich medium containing 25 g ml⁻¹ ampicillin and recovered by lysis of the cells with alkali¹⁸. They were purified by centrifugation to equilibrium in a caesium chloride/ethidium bromide density gradient and purified as described elsewhere¹⁸. The probes were excised by *Hind*III digestion¹⁸. They were further purified by electroelution in water after electrophoresis in 1% agarose gel in 89 mM Tris buffer pH 8.0, 89 mM boric acid, 2 mM EDTA and 0.025 g ml⁻¹ ethidium bromide. They were then purified by chromatography on ELUTIP (Cera Labo) and precipitated with 2 vol. ethanol. The purity and the amount of DNA were estimated from the ultraviolet absorption spectra. Probes were labelled by nick-translation¹⁸ with ³²P-dCTP and ³²P-dTTP to a specific activity of 2.2–2.5 × 10⁸ c.p.m. per g DNA. The DNA from the various monomer fractions was dissolved in 10 mM Tris-HCl pH 8.0, 1 mM EDTA and denatured by heating to 100°C for 10 min. After chilling the samples on ice, an equal volume of 1 M NaOH was added and the solution was incubated at room temperature for 20 min. The DNA samples were then neutralized by adding 0.5 vol. of a solution of 1 M NaCl, 0.3 M sodium citrate, 0.5 M Tris-HCl pH 8.0 and 1 M HCl¹⁸. They were spotted directly onto nitrocellulose paper (BA 83, 0.25 µm, Schleicher & Schuell) which had been treated previously with H₂O and equilibrated with 20 × SSC buffer (1 × SSC buffer is 0.15 M NaCl, 0.015 M trisodium citrate) using a vacuum minifold apparatus (Schleicher & Schuell). The nitrocellulose filters were dried under a lamp and baked for 2 h at 80°C. They were then prehybridized for 24 h at 42°C and hybridized for 65 h at 42°C using the technique described by Thomas²⁰. The blots were exposed to X-ray film for 3 days at -70°C using a Kodak intensifying screen. Total monomers (Tot) were prepared by two successive centrifugations on a 5–30% sucrose gradient for 21 h at 110,000g followed by treatment with RNase and proteinase K and purification by phenol and chloroform¹⁸. Equal amounts of the different DNA samples on the filters were spotted (5 and 7 µg).

other fractions tested (class 1 and 4 monomers). During maturation, the repression of the AFP gene^{8,9} occurs concomitantly with the increase in H1⁰ content^{3,4}. We show here that coding sequences for the AFP gene are preferentially associated with nucleosomes bearing H1⁰. This result reinforces the hypothesis that H1⁰ may be involved at the level of gene expression during development.

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Corrigendum

A bacterial repressor protein or a yeast transcriptional terminator can block upstream activation of a yeast gene

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Nature **312**, 612–615 (1984)

THE fourth paragraph in the Discussion section of this article (p. 615) contained several inaccuracies resulting from the use of upper-case Greek alpha, which looks the same as the upper-case letter A. The paragraph should read:

There is evidence that other yeast genes can be repressed by negative regulatory factors bound between the upstream activator sequence and the TATA region. For example, it is likely that *MATa1* transcription is repressed in diploid yeast by binding of a negative regulatory factor (which is dependent on the expression of *MATa1* and *MATa2*) to a site between the *MATa1* upstream activator sequence and its TATA region¹⁶. *STE6* transcription is repressed by the *MATa2* product, which binds to a specific site in the *STE6* promoter, probably located between the upstream activator sequence and the TATA region (A. Johnson, K. Wilson and I. Herskowitz, personal communication). The repression of *MATa1* and *STE6* is at least 50 times greater than the 10-fold repression we observe for *lexA* operator-containing derivatives of the *GAL1* promoter. We do not understand why these yeast promoters are repressed so efficiently relative to the *lexA* operator-containing *GAL1* promoter.

Sequence similarity between EGF receptor and α_1 -acid glycoprotein

THE human epidermal growth factor (EGF) receptor precursor consists of three domains, an extracellular EGF binding domain, a single transmembrane domain and a cytoplasmic domain. Ullrich *et al.*¹ recently determined the complete amino acid sequence of the molecule and showed that the latter two domains exhibit close similarity in sequence to the entire *v-erb-B* oncogene product, a member of the *src* family. By aligning cysteine residues, they also found repeated structures of ~170 residues within the extracellular domain¹. A similarity matrix comparison², however, revealed the presence of further extensive homology between regions of ~300 residues located at positions 1-274 and 294-615, which cover almost the entire extracellular domain (Fig. 1). Of all the positions compared in the aligned sequences, 28% are occupied by identical residues (gaps were considered as substitutions between different amino acids regardless of their length). Furthermore, a statistical test² showed that the similarity between the aligned sequences is highly significant, with a probability of occurrence by chance of $<10^{-9}$. Thus, the extracellular EGF binding domain con-

sists of tandemly repeated domains which may have evolved by internal duplication. Alignment of their sequences also revealed that each repeat is composed of an N-terminal conserved region and a C-terminal cysteine-rich region.

We subjected the amino acid sequence of the EGF receptor to a computer-assisted search for homology with ~2,500 known sequences compiled in our database and found apparent sequence similarity between the C-terminal half of the EGF binding domain and α_1 -acid glycoprotein (α_1 -AGP), known to be an acute-phase protein³ (Fig. 1). A statistical test shows the similarity to be highly significant, with $P=3 \times 10^{-7}$.

Interestingly, the α_1 -AGP molecule has been shown to exhibit weak sequence homology to immunoglobulin³ as well as to part of the variable domain of the mouse T-cell receptor ($P < 3 \times 10^{-4}$). Thus, it seems likely that the EGF binding domain is indirectly related to the immunoglobulin superfamily. Lack of significant similarity between the N-terminal half of the EGF binding domain and α_1 -AGP may be a result of rapid divergence in the N-terminal half, relative to the C-terminal half, presumably during the earlier evolutionary stage after the separation of the two domains by internal duplication. In addition, the transmembrane segment of this receptor bears a close resemblance

to that of the HLA class II β -chain ($P < 5 \times 10^{-4}$).

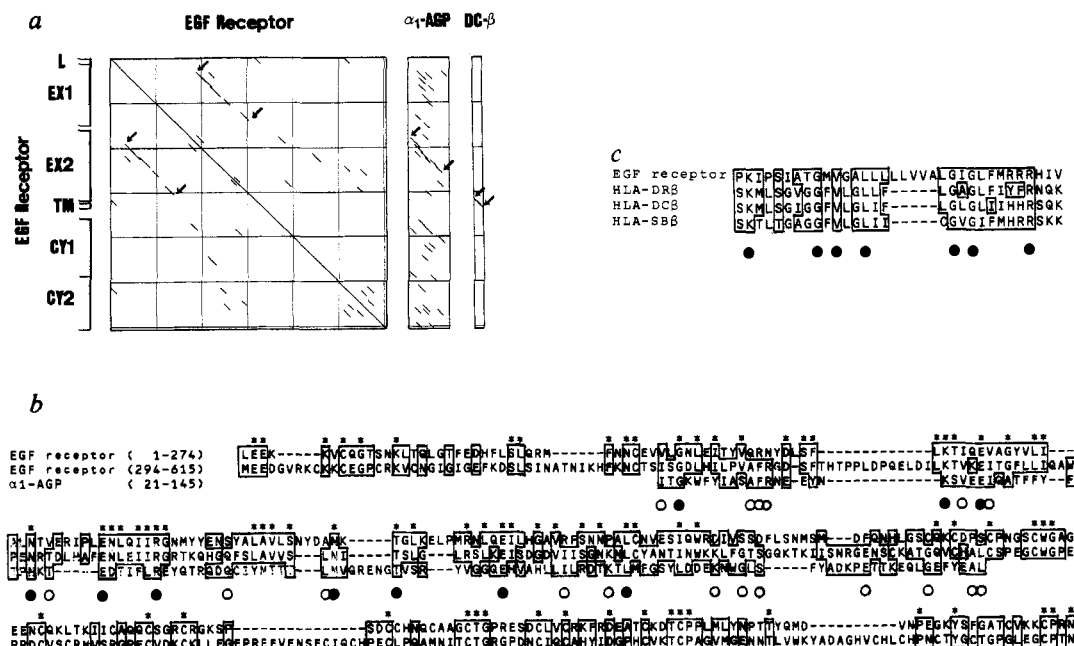
These results, together with evidence that the cytoplasmic domain contains an *src*-related sequence¹, suggest that the extracellular and cytoplasmic domains may have been derived from evolutionarily independent origins and thus that this large receptor gene may have evolved by gene shuffling and partial duplication. We cannot exclude the possibility, however, that an ancestral α_1 -AGP gene was derived from a part of the EGF binding domain.

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Fig. 1 a, Similarity matrix comparisons of amino acid sequence of EGF receptor (ordinate) with the sequences of itself, α_1 -AGP and the transmembrane segment of HLA-DC β -chain (abscissa). A computer program was used to generate diagonal lines indicating segments of 25 residues which show sequence similarity (see ref. 2). L, signal peptide (positions -24 to -1); EX1, EX2, duplicated regions (1-274 and 294-615) of the extracellular EGF binding domain, respectively; TM, the transmembrane segment (622-644); CY1, CY2, *src*-homologous and -non-homologous regions (690-936 and 967-1,186) of the cytoplasmic domain, respectively. Arrows indicate regions where extensive similarities were detected. b, Alignment of amino acid sequences of N-terminal (EX1) and C-terminal (EX2) halves of the EGF binding domain and α_1 -AGP. Boxed residues indicate identities or favoured amino-acid substitutions (see ref. 2). Positions where residues are identical between EX1 and EX2 and between EX2 and α_1 -AGP are marked by * and O, respectively. Invariant residues among the three are indicated by ●. Gaps (—) were introduced to maximize similarity. c, Alignment of transmembrane segments of EGF receptor and HLA class II β -chains. Regions where sequences were aligned correspond to positions 617-650 and 197-225 of the EGF receptor and HLA-DC β , respectively. Boxed residues indicate identities (●) or favoured amino-acid substitutions between EGF receptor and HLA chains.



Stomatal mechanism as the basis of evolution of crassulacean acid metabolism

KEELEY *et al.*¹ have demonstrated clearly that the rare pteridophyte, *Stylites andicola* (Isoetaceae), exhibits a variant of photosynthetic carbon metabolism which has obvious affinities with crassulacean acid metabolism (CAM). They also drew attention to the absence of stomata in *Stylites* and concluded that these findings taken together indicate that this unusual organism does not conform to the hypothesis that CAM may have evolved from stomatal guard cell metabolism².

However, as other members of the Isoetaceae possess stomata, it may be that *Stylites* evolved from ancestors that did possess stomata and although it does not itself possess these structures, it may nevertheless use pathways derived from guard cell metabolism.

Thus the discovery of a CAM-like photosynthetic mechanism in *S. andicola* is not necessarily inconsistent with the evolution of CAM and associated pathways from stomatal guard cell metabolism.

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THE suggestion by Keeley *et al.*¹, supported by Moore², that *Stylites* may represent an important link between aquatic plants and primitive land plants has little foundation in fact. To go further and propose that these plants could be relics of a marginally successful compromise from which present-day terrestrial photosynthetic systems were selected runs contrary to our views on plant evolution. *Stylites*, whose separation from *Isoetes* is questionable, is regarded generally as a descendant of a long line of lycophytes and should not be thought of as primitive plants. Specifically, they most probably evolved from woody-stemmed plants with cormose bases, such as the Carboniferous swamp plant *Chaloneria*. These early lycophytes certainly possessed stomata³. *Isoetes*-like plants existed in the Triassic and probably spread along the coastal plains before moving inland. These are terrestrial species of *Isoetes*, shallow-water-living species (of which some have emergent sporophylls and others are totally submerged) and species living on the bottom of deep-water lakes. This migration into water resulted in the loss of stomatal function in shallow-water-living species (for example, *Isoetes malinverneana*) and the total loss of stomata in the deeper living species (for example, *Isoetes lacustris*)⁴.

The most plausible argument for *Stylites* not possessing stomata is that it lost them through submergence, not that its ancestors never possessed them.

It is true that in its type locality, *Stylites* grows above the water level, but plants have been found repeatedly that grow submerged. The lack of an efficient method of dispersal suggests that the plants have been growing in the same area for a very long time. The substantial elevation of the high Andes in relatively recent geological times could explain both their geographical isolation and the loss of their aquatic habitats.

The fact that both *Stylites* undergo CAM similar to the aquatic species of *Isoetes* is therefore not necessarily surprising. *Stylites* should not be thought of as evidence that CAM did not evolve from stomatal guard cell metabolism. It is more probable that its ability to convert to CAM as it invaded an aquatic environment saved it from extinction.

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KEELEY *ET AL.* REPLY—We agree with Cockburn that, although the presence of CAM in astomatous *Stylites* does not support his theory on the stomatal origin of CAM, it does not necessarily falsify the theory either.

We also agree with Thomas that *Stylites* most reasonably should be included with *Isoetes*, a taxon dating back only to the Triassic. Lycophytes, however, extend back to the Devonian. Although it has been proposed that *Isoetes* represents a reduction from a tree-like lycopod, it is clear that herbaceous lycophytes have been present since the Carboniferous and form the longest unbroken time record of any group of vascular plants¹. We do not believe that *Stylites* (*Isocetes*) *andicola* was the first vascular land plant, but it may represent a model system for how vascular plants invaded seasonally dry terrestrial environments.

The view of the morphological evolution of *Stylites* which Thomas presents agrees with the known fossil record of the Isoetales (see Thomas and Brack-Hanes²). It is also consistent with two arguments proposed by Raven³ as to the physiological significance of morphological attributes in vascular plants, the first of which relates to the presence of xylem tissue in *Stylites*. The probable earliest function for xylem was that of water conduction rather than mechanical support.

Transpiration does not necessarily imply the presence of stomata (compare with the extant *Polytrichum* and the Siluro-Devonian *Cooksonia*), but it does indicate that a phototroph obtains CO₂ from the air rather than the sediment. The second argument relates to the presence of intercellular air spaces which seem to be correlated with the presence, or occurrence at some stage in the plant's evolutionary history, of epidermal pores (Marchantiales) or stomata (sporophytes of vascular plants). These arguments suggest that *Stylites* was derived from transpiring ancestors that possessed stomata and derived CO₂ from the atmosphere rather than the sediment. However, Raven³ noted that "the sequence of acquisition of H₂O-conducting and gas-exchange/regulation mechanisms revealed by the fossil record is generally in accord with teleological expectation, in that the H₂O-conducting system in the absence of the gas-exchange/regulation mechanism would be of more selective advantage than the reverse state of affairs". *Stylites* may represent such a survivor.

Thomas' conjecture about the evolution of *Stylites* is not consistent with our observations. Although it is possible that *Stylites* 'lost' stomata, the argument proposed by Thomas is not plausible; he points out that deep-water *Isoetes* (for example, *I. lacustris*) have totally lost stomata and that the lack of stomata in *Stylites* may derive from a previous aquatic ancestry. Although aquatic *Isoetes* species from deep water are astomatous, all such species so far tested (including *I. macrospora*, considered to be conspecific with *I. lacustris*⁴) will produce stomata on leaves initiated under 'artificial' aerial conditions (J.E.K., unpublished data).

The important feature of the astomatous habit of *Stylites* is the reliance on sediment carbon. Data on *I. triquetra* from the high-altitude paramo of the northern Andes (J.E.K., unpublished) and descriptions of the recently discovered *I. hopei* Croft from the topical alpine of New Guinea suggest that this syndrome, although rare, may be widespread globally in the Isoetaceae.

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Asdic to sonar

R.V. Jones

Seek and Strike: Sonar, Anti-Submarine Warfare and the Royal Navy 1914–54.

By Willem Hackmann.

Her Majesty's Stationery Office: 1984. Pp.487. £15.95.

THE detection and location of submarines in the sea has one factor in common with the analogous processes associated with finding aircraft in the air — the transmission of wave-borne energy. But while the air is a nearly uniform and transparent medium to electromagnetic radiation, and while an aircraft is a palpable discontinuity in that medium, the sea is effectively opaque over almost the entire range of the radio spectrum. It was therefore quickly realized that the detection of submarines would have to depend on acoustic waves — mechanical waves which need transducers for their generation and reception, a further contrast with radar, where radio waves are launched from and received by antennae which are simple extensions of electronic circuitry. As a result, once the problem of aircraft detection was formulated, the radar solution was brilliantly found and exploited to such an extent that in the decade 1935–1945 almost all the essential techniques had been developed.

No such exhilarating story can be told of submarine detection. Instead, it has provided one of the greatest challenges to the application of physics, where ground has been won only by prolonged effort. *Seek and Strike* records what was done in Britain between 1914, when the impact of the U-Boats was first felt, and 1954, when submarine warfare entered a new phase with the launching of the first nuclear submarine — the *USS Nautilus*.

The author, Willem Hackmann, is Assistant Curator of the Museum of the History of Science at Oxford, and he appropriately starts his account with the speculations of Aristotle and Pliny the Younger on the detection of underwater sound by fish. This has its analogy in the use of hydrophones to detect submarines by sound generated by their engines or hydrodynamically by their passage through water; this technique has been developed to detect submarines at ranges of hundreds of miles, but it is limited operationally both by the vagaries of transmission in a medium whose refractive index varies with salinity and temperature, and by a background of other noises generated not only by surface waves and ships, but also by such modest creatures as snapper shrimps.

While hydrophones can give general, and indeed impressive, warnings, ultrasonic echo detection — which gives range as well as bearing — is necessary to locate a submarine precisely enough for it

to be attacked. Hackmann gives the credit for the original development of the method to Paul Langevin, but it may be worth mentioning that James Wilson in his recent biography suggests that some of it should also go to Rutherford.

The author has clearly done a great deal of painstaking work in pursuing the many aspects of a long and involved story, and in refining them into a readable and admirably documented account. Several points emerge from it. First, despite the fact that the Royal Navy appreciated as long ago as 1873 the importance of science when it founded the Royal Naval College at Greenwich to ensure that naval officers would study both science and mathematics, and despite the enthusiasm for science and engineering shown by Admiral John Fisher, few naval officers were prepared to take scientists fully into their confidence, even when faced with the U-Boat threat. Instead of letting scientists such as Rutherford experience the conditions of hunting U-Boats at sea for themselves, the chief naval liaison officer demurred, saying that all that the scientists need be told “was that the enemy submarines were in the sea and that means were required to detect their presence”. Only after the advantages of close cooperation between scientists and serving officers had been demonstrated in air defence in the Second World War were the Services completely won over.

Another feature of the development of Asdic (or Sonar) was the continuous battle between long and short term interests: should greater priority be given to the development of a weapon for tomorrow or to research that might provide a much better weapon the day after tomorrow? The right answer depends on circum-

stances; but in naval weapons, such as Asdic or signalling, the answer was more often than not determined in favour of the short term, because appointments in the Navy have characteristically a two-year duration, and so captains of establishments such as The Signals School tended to favour programmes from which they might expect some concrete result within their term of office.

A further message is the danger of “overselling” a weapon, causing first an unjustified reliance on its performance in operations, and then a loss of confidence when its shortcomings become clear, as exemplified by Churchill’s complaint to the First Sea Lord in 1941 about Asdic — “. . . the failure of our methods, about which so much was proclaimed by the Admiralty before the war”. And yet another is the merit of trying a technique or system “the other way round”, in this case the firing of the depth charges ahead over the bow of the hunting ship instead of dropping them over the stern, which led to an increase in success rate from about 7% to about 30% as a proportion of kills to attacks.

All these lessons, which have relevance far beyond submarine warfare alone, are to be found in the book. Alongside them are such morsels as the attempts of 1915 to train seagulls to hunt for U-Boat periscopes; the renaming of the quartz crystals required for piezoelectric transducers as “asdevite” to avoid disclosing their composition, and the recruitment of a firm of tombstone-makers to cut the crystals; and the fact that even a noisy submarine radiates no more energy than would light a 3-volt torch bulb.

Seek and Strike tells a story of successes tempered with failures in the sustained application of physics to one of the most crucial problems of warfare this century. It was well worth writing — and is well worth reading — both for its significance as history and for the lessons it exemplifies.

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Pioneers of ultrasonic echo ranging; Paul Langevin is fourth from the left, wearing the bowler hat. The photograph was probably taken at Toulon in May 1918.

Breeding discontent

David Pearce

The Plutonium Business and the Spread of the Bomb.

By Walter C. Patterson.

Paladin: 1984. Pp.272. Pbk £2.95.

THERE are, says Walter Patterson, "nuclear people", "plutonium people" and "fast breeder people". Nuclear people believe in the civilian use of nuclear power for electricity. Plutonium people and fast breeder people believe in the original nuclear dream: the widespread use of plutonium — a by-product of burning uranium fuel — as a fuel for fast reactors which can breed more plutonium. Nuclear people need not be plutonium people but they share the same vision, that of a resource with enormous potential. If the resource is there they cannot understand why it should not be used. Yet resource use has costs as well as benefits; nothing comes free.

Patterson sets out to chart one of the costs of plutonium. The civilian nuclear power dream cannot be distinguished from plutonium as a weapons material, despite all the protestations by plutonium people to the contrary. This he does most successfully in an historical overview from Seaborg's identification of plutonium, through the Manhattan Project, to today's aggressive fast breeder programme in France and the limited triumphs and many failures elsewhere. The Super-Phénix is nearing completion in France at a cost of some \$10,000 million. It is jointly owned by France, Italy, Germany, Britain, the Netherlands and Belgium. Patterson is quite clear how such a staggering cost is justified by advocates of the programme — it will provide military plutonium when other supplies have gone. We are all, it seems, helping to finance French nuclear weapons.

Patterson sees no hope in existing safeguards against the spread of military plutonium from civilian sources, whether in the International Atomic Energy Authority or the Non-Proliferation Treaty, the subject of so many ambiguous and confusing interpretations. For the truth is that no one knows collectively how much plutonium exists. One "best guess" for 1982 suggests some 175 tonnes of plutonium "in stock" in civilian sources in the non-communist world (though this appears not to account for high-purity plutonium from Magnox reactors which, if evidence to the Sizewell Inquiry is correct, actually has been diverted to US nuclear weapons). The annual production rate of plutonium is about 40 tonnes. The prospect of control thus seems remote.

In the same light it is easy to see why plutonium people are not concerned about the niceties of nuclear power economics. Any breeder reactor should be credited with the "value" of the plutonium it

produces. By the same token, in the absence of a large breeder programme, all *conventional* nuclear power stations should be credited with the value of the plutonium in their spent fuel. That value lies in the benefit to world security from having nuclear weapons, a value many will find difficult to believe is less than a very large minus number indeed. But if this is the "real" economics of nuclear power why don't plutonium people admit it? The majority may not dispute the positive value of nuclear weapons. The answer may be that most plutonium people, just like most people, do not know the connection between civilian and military plutonium.

The alternative is that they *do* know the link but cannot see how stopping the civilian programme would help. After all, the weapons programme began with reactors dedicated to military plutonium. Electricity was incidental. Patterson offers no guidance on this point. For him it is enough that plutonium production is already out of control. With only a short time to go to the fortieth anniversary of Nagasaki we can only contemplate whether the lapse of time is the truest testament to man's ability to control his fearsome drive to self-destruction, or just an interlude between appalling but vastly different events. □

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Talk of Kyoto

F.S. Rosen.

Progress in Immunology V.

Edited by Y. Yamamura and T. Tada.

Academic: 1984. Pp.1,640. \$110, £85.

IN August, Kyoto is inhospitably hot. Certainly Baedeker, if he ever had reached there, would have found it situated in an unhealthy valley, nonetheless worthy of being reached in slow stages from Edo by the great Tokaido so beautifully depicted in the wood-block prints of Hokusai and Hiroshige. Instead, in August 1983, some 4,000 immunologists were disgorged in haste from the bullet trains, arriving with frequent and clockwork regularity from Osaka and Tokyo, for their triennial self-congratulatory pow-wow.

Over 150 dense, compact summaries of all the symposium lectures have been published in this thick volume. It is a timely and state-of-the-art overview of the whole field of immunology, in just over 1,600 pages of well-set, readable print. To read it from cover to cover is mind boggling, but certainly very informative. Summaries of the dozens of work-shops which took place during the congress are not included but that does not detract from the book; it might have resulted in overkill. Anthony Watkinson recently pointed out (*Nature* 312, 201; 1984) that "the transformation of

a transient three or four days of academic exchange into an enduring collection of reviews can be an invaluable contribution to learning". *Progress in Immunology V* measures up to just that.

On the fourth afternoon of the meeting, a spontaneously exuberant moment occurred when it became apparent that the T cell receptor had at long last been identified in Denver and Boston, in California and Texas. It was one of those rare times when an international congress is ignited by the tinder of a major discovery. Unfortunately, not all of the presentations at that session have been included. While dwelling on the omissions from this volume, it should be noted that there was almost nothing in the congress on immunodeficiency at a time when, among other things, AIDS is creating a stir and yielding information on lymphocytotropic retroviruses. There is also an omission of neuro-immunology at a time when immunology has provided interesting probes for the neurobiologist. And there is barely a nod in the direction of transplantation biology despite some good summaries of the current status of work on the major histocompatibility locus in mouse and man. Maybe all this will be fixed next time around.

Immunologists are returning to their roots with some highly productive and heuristic results. The articles by B.B. Bloom on leprosy, A. Capron on schistosomes and the Nussenzweigs on malaria are vivid demonstrations of how useful immunology can still be to its parent science. The emergence of synthetic vaccines also promises to burgeon into something usefully applicable, and current work in Israel and France on this subject is also well summarized.

The best subject coverage in the book concerns the molecular biology of the immunoglobulin and major histocompatibility genes. This field has moved very rapidly as can be gleaned from the outstanding summaries by D. Baltimore, L. Hood, T. Honjo and others. In contrast there are the still muddy fields of lymphokines, growth factor and other humors. They are desperately in need of being set right.

The high specific gravity of this tome is occasionally relieved by inspirational words, by D.W. Talmage, N.A. Mitchison and R.A. Good, that set the field in perspective. The opening reflections of B. Benacerraf epitomize the message of this meeting: "Nature rewards and yields its magic secrets to the most daringly imaginative. Hard work and dedication, while essential, are not enough". Time will tell if this challenge is to be met at the next congress in Toronto in 1986, and if that meeting too produces such a profusion of progress and excitement. □

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An inside view

Sean C. Solomon

Planetary Interiors.

By William B. Hubbard.

Van Nostrand Reinhold: 1984. Pp.334. \$42.50, £49.75.

THE United States planetary programme is midway through a five-year hiatus in major new observations from spacecraft missions, and an increasing number of American scientists are filling the gap by writing textbooks and monographs on various aspects of planetary science. Hubbard's offering treats the physics and chemistry of planetary interiors, covering all the planets (save Pluto) and most of the larger satellites viewed to date by spacecraft. The text incorporates material up to mid-1983, including results from the Voyager Saturn flyby and Pioneer Venus, but not the Soviet Venera 13-16 missions to Venus. A background in physics, chemistry, astronomy and geology at the level of a senior undergraduate or first-year graduate student is assumed.

The author has attempted a laudably comprehensive treatment of his subject. Discussion of the bulk composition and internal differentiation of each planet is cast in the framework of the composition of the Sun and the evolution of the solar nebula. Particularly thorough chapters are included on equations of state and planetary gravitational fields, both topics on which the author has made a number of original contributions. Accounts of the energy budgets of planetary interiors and the nature of planetary magnetic fields complete the foundation for the final half of the book, a thorough and quantitative inside tour through most of the principal objects of the Solar System.

Hubbard admits at the outset that "a single author has difficulty in writing a current, comprehensive book" on the planets. As if to prove the truth of this assertion, variations in the depth and evenness of coverage betray the background and research interests of the author. There is an implicit bias towards the outer planets, which first becomes apparent in the discussion on equations of state. The only silicate treated is "rock", defined as "that component of [nebular] condensate which is left after formation of an iron core". A chapter on the interior of the Earth is exposed as perfunctory with the opening apology that "this book is about the interiors of other planets"; worse, it contains misleading or incorrect statements about mantle convection, the nature of seismic discontinuities in the upper mantle and the evidence for a solid inner core.

There is a parallel bias towards an astrophysical, rather than a geological, approach to the planets. One telling statistic is that there is only a single photograph of a planetary or satellite surface (of the

Discovery scarp on Mercury). The reader will find only cursory mention of cratering, volcanism, tectonics and lithospheric evolution on the solid planets and satellites. Much of the material on the terrestrial planets appears to have been taken either from the work of the author's colleagues or from a few selected review papers.

Perhaps these shortcomings are visible only from my own geocentric viewpoint. To an alien observer, the large outer planets would initially be the most interesting and an astrophysical approach to their study would be natural; indeed, our discovery and investigation of other planetary systems is, in fact, progressing in this fashion. At least from this cosmic perspective, *Planetary Interiors* is the most current and complete treatise available. □

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Ode to diversity

Lindon J. Eaves

In Praise of Difference: Genetics and Human Affairs.

By Albert Jacquard.

Columbia University Press: 1984. Pp.187. \$20.

"THE essential nature of the Darwinian revolution was... the replacement of a metaphysical view of variation among organisms by a materialistic view". Thus Richard Lewontin (*The Genetic Basis of Evolutionary Change*, 1974, p.4) described the impact of biology on our understanding of life: an impact which has only been magnified by the ability, recently acquired, to extract and hold in our hand the molecular basis of life.

Against this background of the "scientific challenge" to traditional ideas, our acceptance of reproductive technology has already led to a radical adjustment in the norms of society with regard to the control of fertilization and the outcome of pregnancy. How far should we go down this road? In his preface Professor Jacquard writes:

Human endeavor, based on a growing knowledge of both the inanimate and the living world, has become increasingly effective. Why not use this new power to achieve the most tempting objective of all: the improvement of the human species itself?

Popular ignorance of the main scientific ideas and facts involved is the first barrier to democratic decision-making, so Professor Jacquard begins by outlining the elementary reproductive and evolutionary processes as simply as possible and later discusses some of the mechanisms which can lead to the high level of polymorphism not predicted from classical theory in

population genetics. His treatment of these issues is simple and lucid and will be readily comprehensible to the general reader. Having outlined the basic biological issues, Professor Jacquard focuses on subtleties which are sometimes ignored by advocates of a genetic basis to social policy and he tries to give a more balanced view of debatable ideas which are sometimes treated as "facts" in the popular mind. Among the problems he addresses are the "dysgenic" effects of medicine which, he argues, would take place extremely slowly even if they were not offset by such factors as genotype - environment interaction. His statement about consanguinity warrants some qualification. When writing of the origin of rules prohibiting consanguinity, he says (p.50), "the ill effects of consanguinity... are much too slight to be observed empirically". It must be presumed that he is thinking about cousin marriage, since the effects of closer consanguinity on mental retardation, for example, are well documented.

The book points, indirectly, to the separation of population genetics from quantitative genetics which has characterized much of the development of both disciplines. Professor Jacquard's strength is in population genetics. When he turns to quantitative genetics and psychometrics he does not do justice to the best of either subject. For example, he invests great effort in criticizing an additive model for individual differences without asking why such a model often fits the data remarkably well. And he criticizes the concept of general intelligence without addressing the more interesting question of why multiple abilities are correlated.

Bertrand Russell observed that moral considerations could only appear when the truth is known and should not be used to decide what the truth is. There are few moral propositions in the book from which the sensitive reader is likely to dissent. Man's unalienable right to "life, liberty and the pursuit of happiness" is maintained, whether it be given by God, history or DNA. In spite of the growth of knowledge, we do not have all the facts to manipulate the future of the species in the broadest sense. Is this so bad? Professor Jacquard suggests that it is not. The title of his book reflects his belief, expressed in the final chapter, that human diversity, guaranteed by the mechanisms of inheritance and natural selection, is a treasure which should not be sacrificed before any idol of "progress" or "uniformity". This principle extends as much to the fruits of man's intellect as it does to the species itself. The dissemination of scientific ideas, and their open discussion divorced from political influences, is the best insurance that science will remain a guardian of freedom rather than a slave to totalitarianism. □

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Practice of science

Russell Moseley

Philosophy and Sociology of Science: An Introduction.

By Stewart Richards.

Basil Blackwell/Schocken Books: 1984. Pp.210. Hbk £17.50, \$20; pbk £5.95.

An Introduction to Science Studies: The Philosophical and Social Aspects of Science and Technology.

By John Ziman.

Cambridge University Press: 1984. Pp.203. £12.95, \$22.95.

The Experience of Science: An Interdisciplinary Approach.

By Martin Goldstein and Inge Goldstein. *Plenum: 1984. Pp.400. \$22.50, £21.38.*

THE more dogmatic features of an undergraduate education in science (almost as bad as orthodox theology according to Thomas Kuhn) are well known and are enshrined each year in thousands of examination questions which require candidates to demonstrate their competence in solving puzzles within well-established frameworks. Those examining conventions which today we take for granted have not always been so closely observed. Cambridge science students in the 1850s were invited to consider whether there was "a shadow of proof from the ethnographical and physical history of man that any one of his oldest varieties was derived from a quadruminous progenitor" and were urged to state their own opinions "without hesitation" should they differ from those of the examiner. But by and large undergraduate science students have not been encouraged to question either the status of the knowledge acquired in their apprenticeship or, indeed, the nature of the scientific enterprise more generally. Not surprisingly

then, studies which seek to encourage such questioning have met with a mixed reception.

To reduce the possibility of outright rejection it becomes necessary to establish the legitimacy of "science studies" and to convince students of the need to view them as an integral part of a scientific training rather than something which derives from a nineteenth-century belief in the virtues of a liberal education. In this respect students are more likely to take notice if these matters are addressed by *bona fide* scientists, and all three of the books reviewed here share this advantage. Credibility in students' eyes may be further enhanced by claims for the utility of science studies, and each book contains a justification for such teaching over and above "metascience" (as Ziman calls it) for its own sake. Thus Richards begins with a recollection of the value to his later career of an undergraduate course in the history and philosophy of science, while Ziman sees "a possible guide to action in scientific research, in industrial management, in political administration, and in public affairs". Once credibility has been established a major problem remaining for the would-be teacher of science studies is the choice of material — there is no agreed core curriculum here but a spectrum extending from the philosophy of science to the economics of technological innovation. This is a difficulty which confronts any author writing a book on science studies, and the problem of providing material and a structure which will be sufficiently flexible to find use in a broad range of courses has no easy solution. Since all three books make claims to be suitable undergraduate texts, their success in this respect deserves careful consideration.

An additional obstacle to be overcome stems from the constantly growing specialist literature generated by those historians, philosophers, sociologists, economists and political scientists who contribute to the science studies area. Both Richards and Ziman are acutely aware of this — their titles make it clear that we are dealing with "an introduction" — and both authors offer their work as a guide to an interpretation of the wider literature and as a means of presenting themes which they identify as being of particular importance. For a reviewer this poses a problem. To engage in a discussion of the authors' interpretation of debates in the philosophy of science, the sociology of science, science policy and so on, would be an undertaking which would result in another (introductory) text. Instead it seems preferable to focus on the appropriateness of the particular guided tours we are being offered in the light of their stated purpose.

The routes taken by both Ziman and Richards are, at first glance, very similar, starting with a broad look at selected features of the philosophy of science, moving on to the sociology of science and science policy, bringing us finally to more

general cultural matters. Both tours finish rather abruptly, with neither guide attempting any recapitulation of those features which were of special interest — presumably they are taken to be self-evident if the tourist has been paying attention. Despite the apparent similarities there are some significant differences. On the whole the Ziman route is the more stimulating, revealing some unexpected views, striking out on paths only recently discovered and generally traversing the terrain at a faster pace. The Richards party will certainly get their money's worth, but will miss some of the newer landmarks, will avoid some of the higher slopes, and generally travel more sedately.

Ziman is noticeably the stronger in his discussions of recent work in the sociology of science and is more successful in linking his themes throughout the book. Richards more clearly divides his work into "Methods and Philosophies of Science" and "Interactions of Science and Society". The least satisfactory part of both books is that dealing with the organization of research and development and technological innovation — at this point the tourist may find his or her attention wandering (to be fair, neither author sets out to treat the broad area of science, technology and society comprehensively, but questions concerning the economics of research and development and technical change are a part of science studies, and one for which students often show considerable interest). Similarly, the history of science is allowed little more than a token appearance. Students using either of these books will need to be reminded that the Ziman and Richards routes are not the only ways of seeing the landscape and that others have been mapped out.

The Goldsteins offer an alternative approach, and the intended use of their book as an interdisciplinary introduction to science means that metascientific questions are viewed from a different perspective. There is, in addition, a welcome historical dimension introduced through case-studies, those commodities which are always so eagerly sought by teachers of science studies. As with the other two books it would be possible to direct students to specific sections rather than treat the text as a whole. Indeed, some selection of material is probably inevitable since few science studies teachers have sufficient time at their disposal to permit more than a sampling of the range of topics introduced in the three books, let alone persuade students to read into the chosen areas as suggested. Stimulating as these guided tours may be, the problem remains of translating them into the one or two hour a week teaching session typically allowed for such studies in the science curriculum. □

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Visitors to the cave paintings of Lascaux in France, shortly after the cave's rediscovery in 1940. The picture is reproduced from the new paperback edition of *The Creative Explosion*, by John Pfeiffer, published by Cornell University Press. For review see *Nature* 302, 764 (1983).